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The Enzymology and Genetics of Dna Repair in Drosophila Melanogaster (Ap Endonucleases, Oregon-R, Mus(2)201, Cleavage Mechanisms).

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THE ENZYMOLOGY AND GENETICS OF DNA REPAIR IN *DROSOPHILA MELANOGASTER*

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Biochemistry

Interdepartmental Studies in Genetics

by
Andrea Lynn Spiering
B.A., Rice University, 1978
August 1985

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I would like to thank Dr. Andy Deutsch for his help and friendship. Without his moral and academic support, my stay at LSU would have been more difficult.

I would also like to thank my family and friends for their support during my academic career. A graduate education is something you pursue on your own, but the support and understanding of those close to you make it a more pleasant experience. At times I know it was difficult for them to understand why I was here, but overall, I do believe it was worthwhile. I can honestly say that I have enjoyed my time as a graduate student, but enough is enough!

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AP	apurinic/apyrimidinic
APyr	apyrimidinic
ATP	adenosine triphosphate
BAP	bacterial alkaline phosphatase
BSA	bovine serum albumin
cpm	counts per minute
dATP	deoxyadenosine triphosphate
dNTPs	deoxyribonucleotide triphosphates
dsDNA	double-stranded DNA
fmol	10^{-15} moles
$NaPP_i$	sodium pyrophosphate
MMS	methyl methanesulfonate
NP-40	Nonidet P-40
NT	nontreated
OH	hydroxyl group
P	phosphate
PAGE	polyacrylamide gel electrophoresis
PD	pyrimidine dimer
pmol	10^{-12} moles
rpm	revolutions per minute
SDS	sodium dodecyl sulfate
ssDNA	single-stranded DNA
TCA	trichloroacetic acid

ABSTRACT

The work presented herein investigates the repair of apurinic/apyrimidinic (AP) sites in Drosophila melanogaster. Two different AP endonucleases were isolated differing in pH optima, reaction requirements, and associated activities. AP endonuclease I flows through phosphocellulose, has a pH optimum of 6.5, and has no obvious cofactor requirements for the incision of AP DNA. AP endonuclease I additionally has associated non-specific endonuclease activity, inseparable throughout the purification. AP endonuclease II is retained by phosphocellulose, eluting between 200-400 mM potassium phosphate. This enzyme has an absolute requirement for magnesium, a pH optimum of 7.0, and no detectable associated enzyme activities. Both enzymes cleave the phosphodiester backbone of AP DNA, with no activity on alkylated, heat-denatured, UV, or OsO_4-treated DNA substrates. Upon further purification of the enzymes, Sephadex G-100 analysis gave molecular weight estimates of 65-70,000 daltons. Moreover, the two D. melanogaster AP endonucleases and E. coli endonuclease IV were found to cross-react with an antibody prepared against a human AP endonuclease, pointing to conservation of some antigenic determinants through evolution. Using this cross-reactivity, immunoblots of SDS-PAGE gave estimates of 68,000 daltons for AP endonuclease I and 64,000 daltons for AP endonuclease II.

The precise incision position relative to the AP site was also determined for each D. melanogaster endonuclease. AP endonuclease I cleaves DNA 3' to the AP site, producing a 3'-deoxyribose-P and a 5'-OH. AP endonuclease II also nicks DNA 3' to the AP site, but leaves a 3'-deoxyribose-OH and a 5'-P terminus. In the context of DNA repair in general, the results presented here provide the first example of non-complementary classes of AP endonucleases and additionally identifies the first AP endonuclease that leaves a 3'-P terminus.

The data presented will hopefully open the way for analysis of the mutagen sensitive D. melanogaster stocks. Toward that end, data is presented on AP DNA repair in two D. melanogaster strains deficient for base excision repair. Analysis of these mutagen sensitive strains should lead to a better understanding of the role of AP endonucleases in eukaryotic DNA repair.

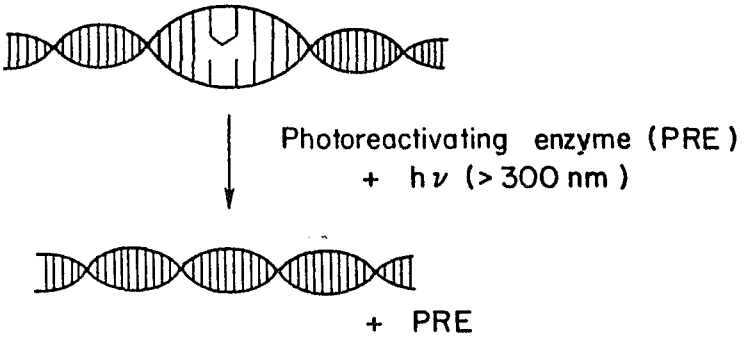
I. INTRODUCTION

DNA repair is essential for the restoration of genomic integrity following DNA damage. Damage to DNA in vivo may be instigated by a variety of chemical, physical, and environmental stimuli. Many chemical alkylating agents have been found to react with individual bases in DNA, leading to possible mutation by the formation of transitions and transversions if not repaired. Additionally, alkylation of DNA bases results in an increased lability of the glycosylic bond in DNA. Physical insults, such as UV irradiation, lead to the formation of pyrimidine dimers, [6-4] lesions, and/or 5-thyminyl-5-6-dihydrothymine. Ionizing radiation causes crosslinking, strand breaks, and thymine glycol formation. Other agents responsible for crosslinking include nitrous acid, mustard gases, and psoralens. Anti-tumor drugs containing cis-diamminedichloroplatinum (II) (Rosenberg's compound) have also been associated with DNA crosslinking. In the absence of external stimuli, misincorporation of nucleotides during DNA replication constitutes a source of error. S-adenosyl-L-methionine has also been shown to methylate DNA in vitro (Rydberg and Lindahl, 1982), possibly for cellular modification of purines in DNA. Additionally, DNA may undergo spontaneous deamination by which cytosine → uracil, adenine → hypoxanthine, and guanine → xanthine, leading to transition mutations or uracil in DNA.

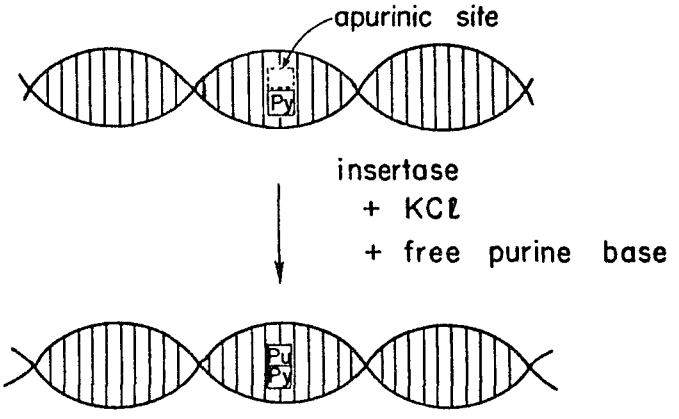
In addition to the above stimuli, spontaneous base loss via protonation of the base and cleavage of the glycosyl bond may occur, resulting in an apurinic or apyrimidinic site. This occurs, in mammalian cells, at an estimated rate of 5,000-10,000 purines or 200-500 pyrimidines per 20 h generation time (Lindahl and Nyberg, 1972). As described above, the possibility for DNA damage appears overwhelming. Regardless of the method by which DNA lesions occur, these potential errors must either be repaired or tolerated. If not repaired, DNA alterations may lead to transitions, transversions, frameshift mutations, or to complete blockage of replication or transcription. Since an observed mutation frequency of only 10^{-9} to 10^{-12} mutations per nucleotide is observed (Drake, 1969), repair rather than tolerance must regularly occur.

How does an organism accomplish DNA repair? Three pathways are believed to account for the repair of the many lesions seen in DNA. These pathways are: base excision repair, nucleotide excision repair, and lesion reversion. [For reviews see Lindahl, 1979, 1982; Laval and Laval, 1980; Grossman et al., 1978; Friedberg, 1984.] Lesion reversion is accomplished via a one-step reaction -- therefore theoretically the more "error-free" method of repair. At 200-300 nm light, pyrimidine dimers undergo photo-reversal without any enzymatic catalysis. Above 300 nm light, photoreactivating enzyme (PRE or photolyase) completely reverses pyrimidine dimer formation (Sutherland, 1982; Fig. 1a) without the removal of either of the involved bases or disruption of the phosphodiester backbone. A second example of lesion reversion is the repair of apurinic sites by insertase (Deutsch and Linn, 1979; Livneh et al., 1979). This enzyme

A. Photoreactivation of pyrimidine dimers



B. Insertion of purines into apurinic sites



C. Demethylation of alkylated bases (O^6meG)

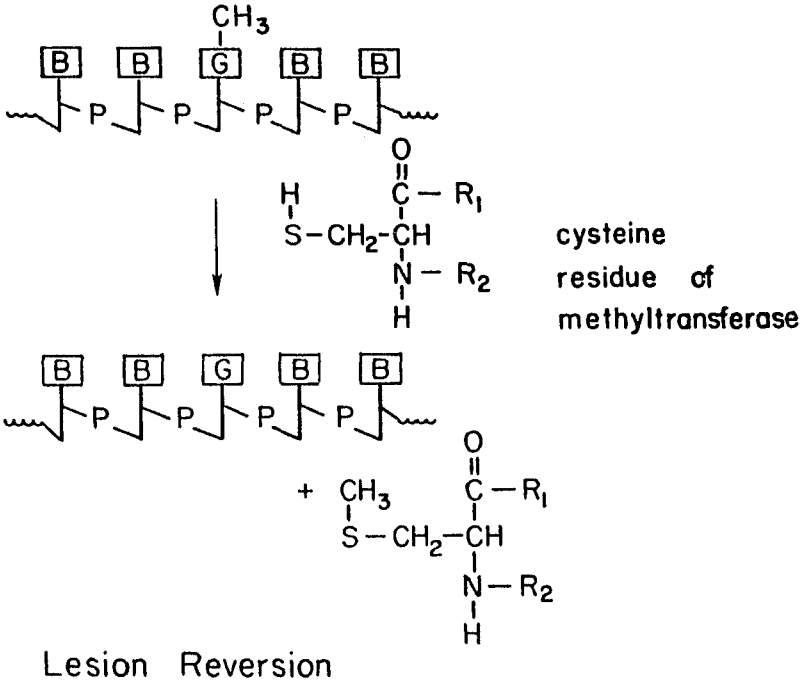


Figure 1 : Lesion Reversion

is capable of reinsertion of the correct purine into duplex apurinic DNA (Fig. 1b). A third and more recent example of lesion reversion is the discovery of a class of proteins known as methyltransferases (Karran et al., 1979). The alkyl group is transferred from the base to a cysteine residue of the protein, repairing the DNA without the removal of the affected base (Fig. 1c). Unlike the previous two examples of lesion reversion, methyltransferases are suicidal proteins. This pathway has been seen for the removal of O^6methylguanine from DNA in E. coli (Olsson and Lindahl, 1980), B. subtilis (Behrens et al., 1983), mouse liver (Bogden et al., 1981), and human cell lines (Brent, 1979). These proteins may be constitutively expressed at low levels; the prokaryotic enzymes are inducible by exposure to alkylating agents. Other alkylated bases such as N^7methylguanine and ^{3}methyladenine may also undergo lesion reversion via this method in D. melanogaster (Green and Deutsch, 1983).

Unlike lesion reversion, nucleotide excision repair is a multi-step process (Fig. 2). The lesion is recognized by a damage-specific DNA endonuclease which cleaves in the vicinity of the damage, creating a nick in the duplex DNA. This is followed by 5'→3' exonuclease activity, creating a small gap in the DNA (hence nucleotide excision). DNA synthesis by DNA polymerase then returns the DNA to its original state, with the remaining 5'-P, 3'-OH sealed by DNA ligase. E. coli endonuclease V initiates repair for a variety of lesions via this mechanism. Alternately, the damage specific endonuclease may cleave on either side of the lesion, incising and excising the damage simultaneously. Again, DNA polymerase and DNA

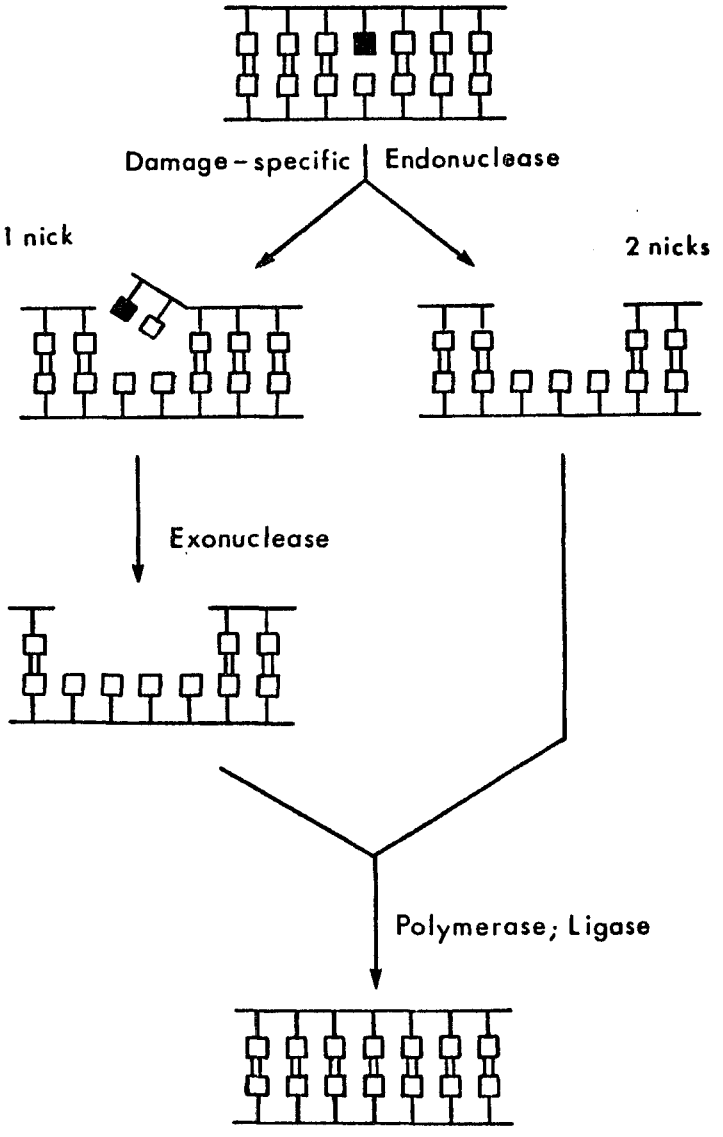


Figure 2. Nucleotide excision repair pathway.

ligase complete the repair. The uvrABC enzyme is believed to act in this manner to repair pyrimidine dimers (Friedberg, 1984). A nick is made 7-8 nucleotides 5' to the lesion and 3 nucleotides 3' to the lesion, creating a 12 nucleotide gap in the duplex DNA. DNA polymerase may then resynthesize the correct nucleotides and DNA ligase seal the remaining nick. Within a single pathway -- nucleotide excision repair -- multiple mechanisms for DNA repair exist.

Base excision repair involves the coordinated efforts of four enzyme activities: DNA glycosylase, AP endonuclease, DNA polymerase, and DNA ligase (Fig. 3). Initially, a specific DNA glycosylase recognizes the altered base (Table I), cleaving the sugar-base glycosyl bond and releasing the free base. This results in a secondary lesion, a baseless site (hence base excision). This baseless site is then recognized by a second activity, an apurinic/apyrimidinic (AP) endonuclease. This enzyme nicks the phosphodiester backbone in the vicinity of the AP site. DNA polymerase, with associated exonuclease activity, reinstates the appropriate nucleotides. DNA ligase completes the repair, returning the DNA to its original conformation.

From the above descriptions of DNA repair, one can see the complexity of repair for a single lesion. Not only are there multiple pathways, but within each pathway there are further mechanistic variations. The AP site is the lesion of interest in this study. This lesion occurs by the action of DNA glycosylases, spontaneous depurination or depyrimidination, or as a secondary effect of other types of DNA damage. AP sites are able to be repaired by either lesion reversion or by steps coincident with base excision repair.

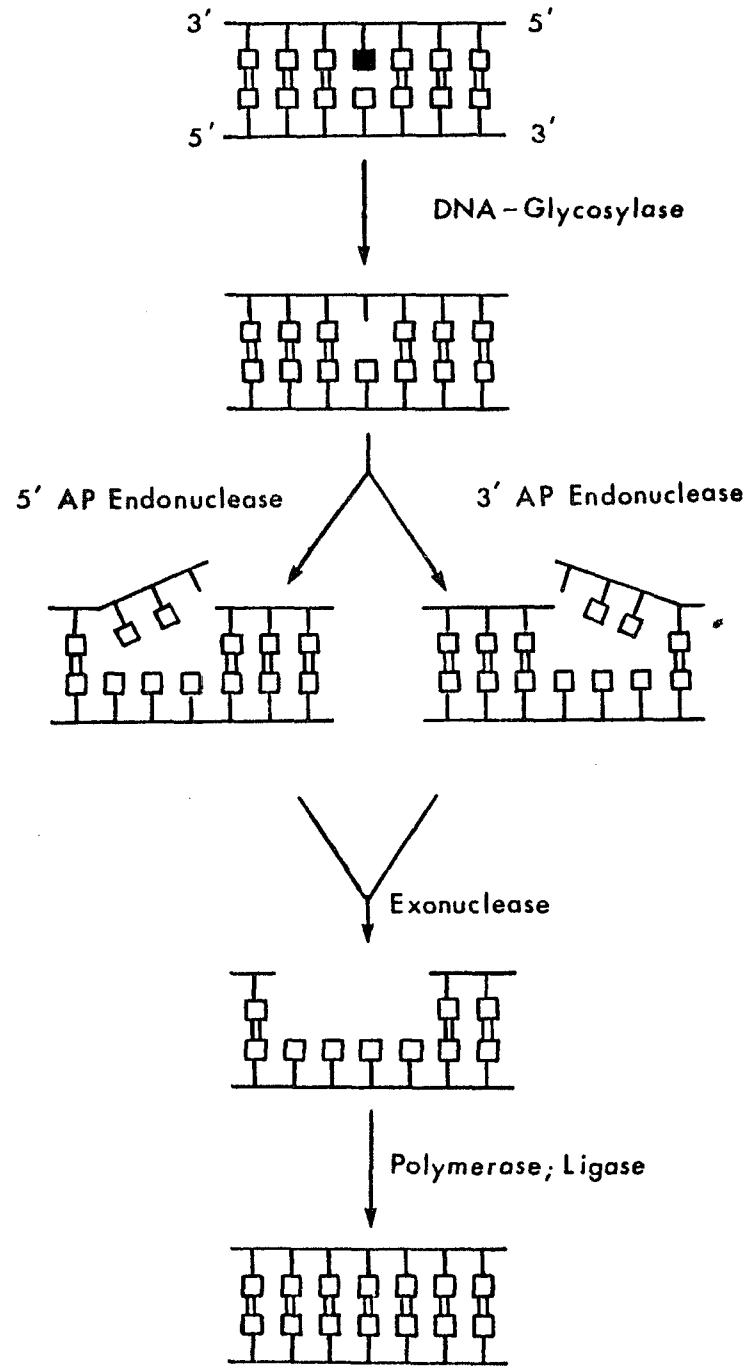


Figure 3. Base excision repair pathway.

Table I. DNA-glycosylases.

Substrate	Enzyme	Baseless site
Formamido-pyrimidine	FaPy DNA-glycosylase	Apurinic
Hypoxanthine	HX DNA-glycosylase	Apurinic
^{3}methyladenine	^{3}meA DNA-glycosylase	Apurinic
^{7}methylguanine	^{7}meG DNA-glycosylase	Apurinic
^{3}methylguanine	^{3}meG DNA-glycosylase	Apurinic
5,6-Hydrated thymine	5,6-HT DNA-glycosylase	Apyrimidinic
Pyrimidine dimers	PD DNA-glycosylase	Apyrimidinic
Uracil	Ura DNA-glycosylase	Apyrimidinic
Urea	Urea DNA-glycosylase	Apyrimidinic

The exact conditions by which an organism selects one repair pathway over another or one particular enzyme over other available enzymes is not well understood. As such, the AP site represents an excellent lesion for the study of DNA repair in D. melanogaster, exhibiting the complexity of repair by a variety of mechanisms within a single test system.

AP endonucleases.

AP endonucleases appear to be ubiquitous, with some organisms containing multiple forms of AP endonuclease. Various AP endonucleases which have been isolated within a single organism are differentiated by physical properties, cofactor requirements, associated activities, and activity toward alternate substrates. Some of the known AP endonucleases and their characteristics are summarized in Table II. These generally fall into two categories -- those which have no associated enzymatic activities and those which do. Many AP endonucleases have been isolated which recognize only apurinic/apyrimidinic sites. Other AP endonucleases recognize substrates in addition to AP sites. Several AP endonucleases have been found which contain associated DNA glycosylase activities and/or exonuclease activities, perhaps enabling them to carry out base excision repair more efficiently. Additionally, not all species of AP endonucleases within an organism have the same affinity for their substrates, differing as much as 10-fold in their K_m's (Kuhnlein et al., 1976)

Another major distinguishing factor between different AP endonucleases within an organism is the position of the cleavage event relative to the AP site. Figure 4 shows four theoretical classes of

Table II. Deoxyribonucleases acting on AP DNA

Prokaryotic Deoxyribonucleases	Substrate	Associated Activities	Reference
E. coli endonuclease III	AP dsDNA alkyl-DNA UV DNA (not PD)	5,6-HT DNA-glycosylase	Gates and Linn, 1977
endonuclease IV	AP dsDNA	-----	Ljungquist, 1977
endonuclease V	AP DNA alkyl-DNA OsO_4-DNA Uracil DNA UV DNA	-----	Gates and Linn, 1977
endonuclease VI	AP DNA	exonuclease III (3'-5') phosphatase RNAse H	Gossard and Verly, 1975 Yajko and Weiss, 1975
endonuclease VII	APyr ssDNA	-----	Friedberg, et al., 1981
T4 UV endonuclease	AP dsDNA	PD DNA-glycosylase	Nakabeppu and Sekiguchi, 1981
M. luteus UV endonuclease	AP DNA	PD DNA-glycosylase	Grafstrom, et al., 1982
endonuclease A	AP DNA alkyl-DNA carcinogen-modified DNA	-----	Hecht and Thielman, 1978
endonuclease B	AP DNA	-----	Pierre and Laval, 1980
H. influenzae AP endonuclease	AP DNA	exonuclease (3'-5')	Clements, et al., 1978
B. stearothermophilus endonuclease	AP DNA	-----	Bibor and Verly, 1978

Table II. Deoxyribonucleases acting on AP DNA (continued)

Eukaroytic Deoxyribonucleases	Substrate	Associated Activities	Reference
C. reinhardi			
AP endonuclease I	AP DNA	-----	Frost and Small, 1984
AP endonuclease II	AP DNA	-----	Frost and Small, 1984
S. cerevisiae			
AP endonuclease A	AP DNA	-----	Akhmedov, et al., 1982
AP endonuclease B	AP DNA	-----	Akhmedov, et al., 1982
P. multifloris	AP dsDNA	-----	Thibodeau and Verly, 1977
D. carota AP endonuclease	AP DNA	-----	Talpaert-Borle and Liuzzi, 1982
Calf			
liver, AP endonuclease	AP dsDNA	-----	Kuebler and Goldthwait, 1977
thymus, AP endonuclease	AP dsDNA	-----	Ljungquist, et al., 1975
Rat			
liver, AP endonuclease	AP dsDNA	-----	Verly and Paquette, 1973
Human			
HeLa, AP endonuclease I	AP dsDNA	----	Kane and Linn, 1981
Fibroblast, AP endonuclease I	AP dsDNA	-----	Linsley, et al., 1977
AP endonuclease II	AP dsDNA		
Lymphoblasts	AP dsDNA	5'-3' exonuclease	Bose, et al., 1978

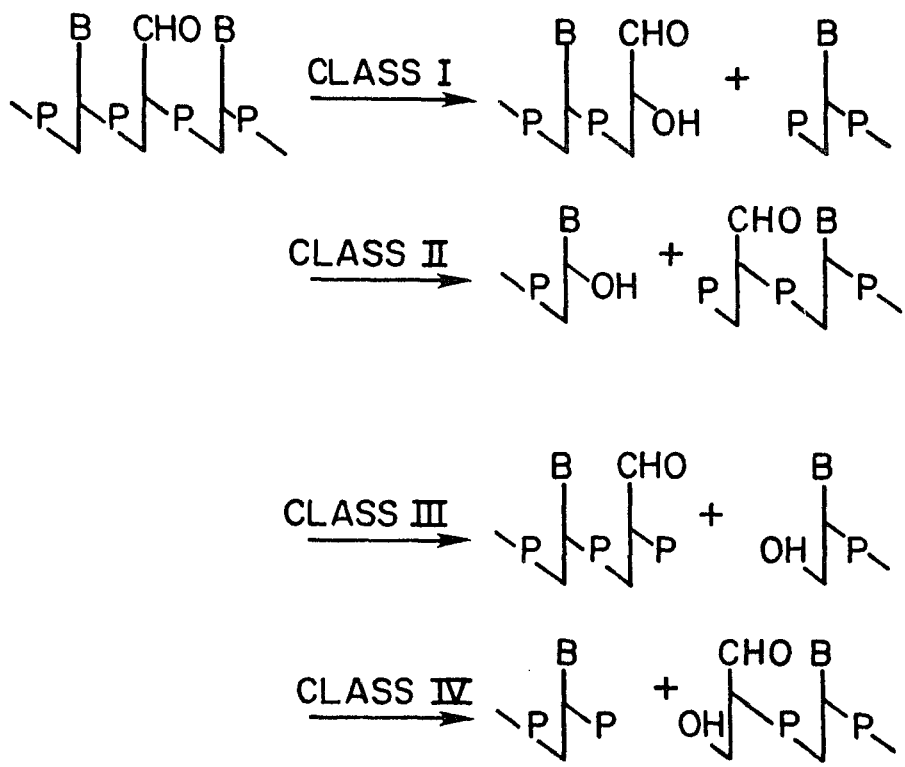


Figure 4. Theoretical cleavage sites for AP endonucleases.

AP endonucleases defined by Mosbaugh and Linn (1980). AP endonucleases have been identified which leave either a 3'-deoxyribose and 5'-phosphomonoester (Class I) or a 3'-OH and a 5'-deoxyribose (Class II). DNA polymerase requires a 3'-OH to bind to the DNA prior to DNA synthesis. Cleavage of the DNA by a Class I or II AP endonuclease primes the DNA for DNA polymerase binding. Class II AP endonuclease cleavage provides for efficient repair of the lesion. Class I AP endonuclease cleavage, however, is less efficient due to the required removal of the 3'-deoxyribose for complete repair of the AP site. The limitations of Class I cleavage may be overcome by the concerted action of the two classes of AP endonuclease for the complete removal of the baseless residue (Fig. 5). This leaves a gap of only one nucleotide as well as an efficient primer for polymerase. Many AP endonucleases have been classified according to cleavage products (Table III). Class IV AP endonucleases, to date, have not been identified. It is interesting that until this study, organisms containing Class I AP endonucleases had either associated glycosylase activity or an additional Class II AP endonuclease.

The work presented here investigates the repair of AP sites in Drosophila melanogaster. The distinct developmental stages, short life span, and available mutagen sensitive stocks make this genetically defined eukaryote the system of choice to link prokaryote and mammalian DNA repair studies. An analysis of AP endonucleases in D. melanogaster was undertaken to identify and characterize any enzyme species present in the wild-type organism. Two different AP endonucleases were isolated (Spiering and Deutsch, 1981), separable on phosphocellulose chromatography. The two AP endonucleases differ in

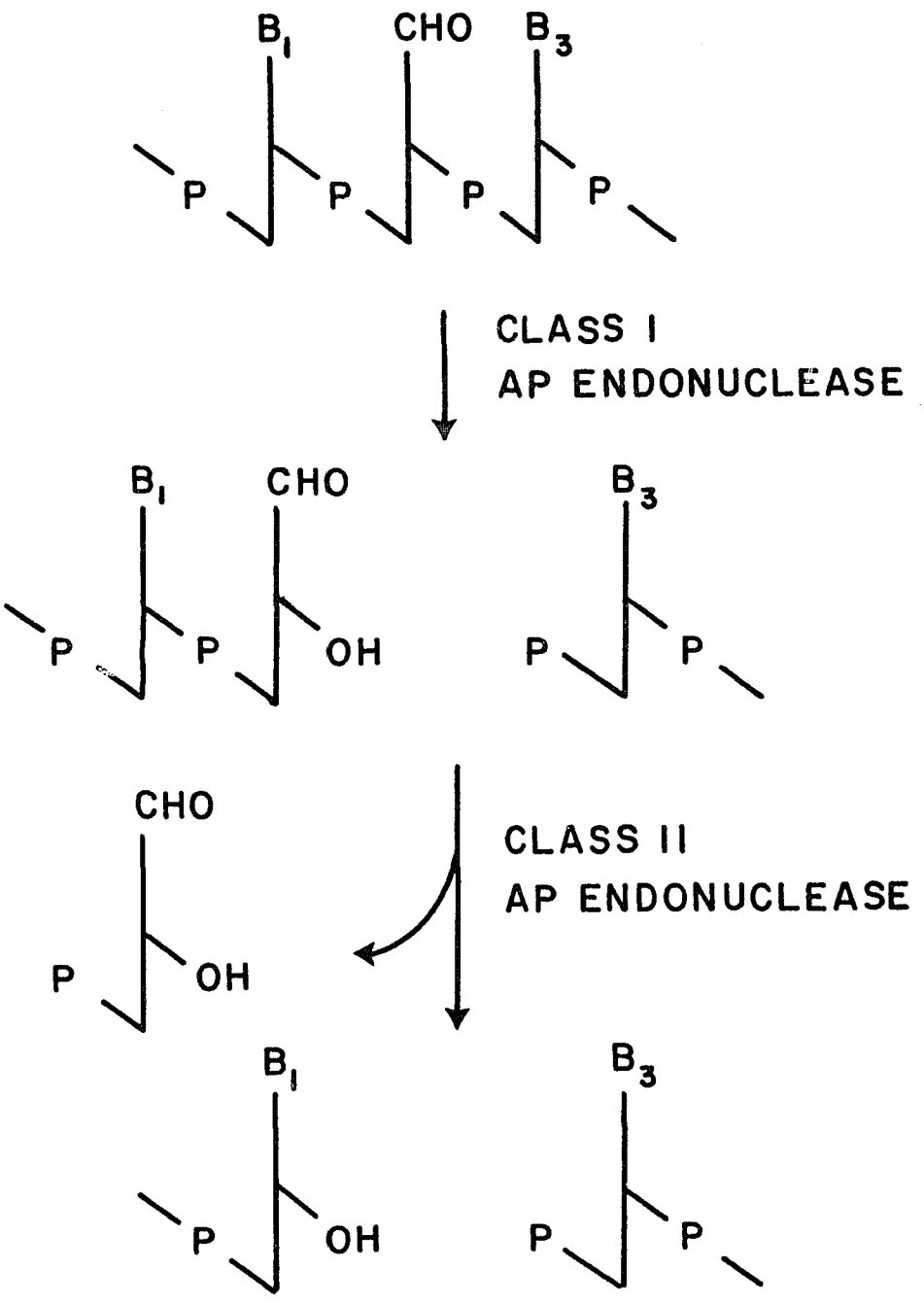


Figure 5. Concerted action of Class I and II AP endonucleases.

Table III. AP endonucleases of known cleavage class.

Deoxyribonuclease	Class	Reference
E. coli		
endonuclease III	I	Warner *et al.*, 1980
endonuclease IV	II	Warner *et al.*, 1980
endonuclease VI	II	Warner *et al.*, 1980
T4 UV endonuclease	I	Demple and Linn, 1980
M. luteus UV endonuclease	I	Hecht and Thielman, 1978
Human fibroblast		
AP endonuclease I	I	Mosbaugh and Linn, 1980
AP endonuclease II	II	Mosbaugh and Linn, 1980
Human placenta AP endonuclease	II	Linsley *et al.*, 1977
HeLa AP endonuclease	II	Kane and Linn, 1981

pH optima, reaction requirements, and associated activities. AP endonuclease I flows through phosphocellulose, has a pH optimum of 6.5, and requires no cofactors for incision of AP DNA. AP endonuclease I additionally has associated non-specific endonuclease activity, inseparable throughout the purification. AP endonuclease II is retained by phosphocellulose, eluting between 200-400 mM potassium phosphate. This enzyme has an absolute requirement for magnesium, a pH optimum of 7.0, and no detectable associated enzyme activities. Both enzymes are specific or partially specific for apurinic-apyrimidinic DNA, with no detectable activity on alkylated, heat-denatured, UV, or OsO_4-treated substrates. Upon further purification of the enzymes, Sephadex G-100 analysis gave molecular weight estimates of 60-70,000 daltons. Moreover, the two D. melanogaster AP endonucleases and E. coli endonuclease IV were found to cross-react with an antibody prepared towards HeLa AP endonuclease, pointing to conservation of some antigenic determinants through evolution. The use of the antibody prepared toward HeLa AP endonuclease also allowed for molecular weight estimates by Western blot analysis. AP endonuclease I has a subunit molecular weight of 68,000 daltons; AP endonuclease II has an apparent subunit molecular weight of 64,000.

The precise incision position of each endonuclease relative to the AP site was also determined. AP endonuclease I cleaves DNA 3' to the AP site, producing a unique 3'-deoxyribose-P and a 5'-OH. AP endonuclease II also nicks DNA 3' to the AP site, leaving a 3'-deoxyribose-OH and a 5'-P. D. melanogaster AP endonuclease I, from results presented herein, should be classified as a Class III AP

endonuclease, while D. melanogaster AP endonuclease II is classified as a Class I AP endonuclease. This is the first known occurrence of a Class I endonuclease without a concurrent Class II endonuclease, and furthermore, is the first example of an organism containing a Class III enzyme for the repair of depurinated DNA.

The data presented here will hopefully open the way for analysis of available mutagen sensitive D. melanogaster stocks. Toward that end, data is presented on AP DNA repair in two Drosophila mutants. Analysis of these mutagen sensitive strains should lead to a better understanding of the role of AP endonucleases in eukaryotic DNA repair.

II. PURIFICATION AND CHARACTERIZATION OF TWO APURINIC/APYRIMIDINIC ENDONUCLEASES FROM *DROSOPHILA MELANOGASTER*

MATERIALS AND METHODS

Materials. The protease inhibitor Aprotinin, agarose, bovine serum albumin, Coomassie Brilliant Blue R, glycerol, harmane (1-methyl-9H-pyrido-[3,4-b]indole), Sephadex G-100, streptomycin sulfate, and Triton X-100 were obtained from Sigma Chemical Company. Nitex mesh was obtained from Tetko, Inc. [^{3}H]-Thymidine, specific activity 50-75 Ci/mmol, was purchased from New England Nuclear. Nitrocellulose filters, type BA85, were obtained from Schliecher and Schuell for use in the filter binding assay. Nitrocellulose membranes used for immunological analysis were obtained from Millipore. Phosphocellulose P-11 was purchased from Whatman. Affi-Gel blue and the Bradford Protein Assay reagent kit were purchased from Bio-Rad. DEAE-cellulose, Type 40, was obtained from Brown Company. Centricon-10 microconcentrators were purchased from Amicon.

Endonuclease IV and uracil-DNA glycosylase were purified from Escherichia coli as described by Deutsch and Spiering (1982). HeLa AP endonuclease and antibody prepared toward HeLa AP endonuclease were generous gifts of C. Kane and S. Linn. [^{125}I]protein A from Staphylococcus aureus was provided by M. West and R. C. Montelaro.

Drosophila melanogaster strains. Two wild-type strains were used, Canton-S and Oregon-R. These stocks were maintained in the laboratory of W. R. Lee, Dept. Zoology, LSU. Stocks were routinely kept on corn meal/agar media at 25°C.

D. melanogaster embryo collection and storage. Population cages were established with 20-40,000 3-4 day old adult D. melanogaster as

described by Spiering and Deutsch (1981). Embryos were collected at either 8 or 12 hour intervals by gentle removal from the food surface onto pre-weighed Nitex HD 140 mesh. Embryos were washed in 0.1% Triton X-100/0.7% NaCl followed by repeated washing in deionized water. After transfer to 15-ml polyallomer tubes (less than 0.8 g wet weight embryo/tube), the embryos were washed with 70% ethanol. The embryos were then dechorionated by addition of 50% NaClO and centrifugation at 1000 rpm in a clinical centrifuge for two minutes, total time. This treatment was terminated by repeated washing in 0.1% Triton X-100/0.7% NaCl to remove the NaClO and any remaining debris. Next, the embryos were washed with 50 mM potassium phosphate, pH 7.5/ 1 mM EDTA and transferred to a sterile 1.5-ml eppendorf tube. The embryos were covered with a solution of 50 mM potassium phosphate, pH 7.5/1 mM EDTA and 25 µl Aprotinin per ml buffer. After 12 h at -20°C, the embryos were routinely transferred to liquid nitrogen for storage.

Nucleic acids. PM2 [^{3}H]DNA was prepared according to the method of Kuhnlein, et al. (1976) with the following modifications. [^{3}H]-Thymidine was used at 1 mCi/liter and bacteria (5 x 10^8/ml) were infected at a multiplicity of infection (moi) of 2. Unlabelled PM2 DNA was isolated following the same protocol, with the omission of the [^{3}H]-thymidine. PM2 DNA was bisulfite-treated by the method of Lindahl et al. (1977). Osmium tetroxide-treatment of PM2 DNA was according to Gates and Linn (1977). Methyl methanesulfonate-treatment of PM2 DNA was as described by Spiering and Deutsch (1981). UV treatment of PM2 DNA was according to the procedure of Petranovic et al. (1978). DNA was depurinated at pH 5.2, 70°C, for 15 min to yield

2.2 AP sites per DNA molecule (Kuhnlein et al., 1976). Calf thymus DNA was purchased from Sigma.

Apurinic endonuclease (nicking) assay. Assay mixtures (0.05 ml) contained 25 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 60-200 fmol depurinated PM2 [^{3}H]DNA and an aliquot of protein. AP endonuclease I is routinely assayed in the absence of divalent cations at pH 6.5; AP endonuclease II is routinely assayed at pH 7.0 with 10 mM $MgCl_2$. Incubations were for 5 min at 37°C. Detection and quantitation of nicks produced follows the procedure of Kuhnlein et al. (1979), assuming a Poisson distribution of nicks/DNA molecule. One unit of endonuclease activity represents 1 pmol of nicks per minute. Specific AP endonuclease activity was determined through comparison of activities on AP versus nontreated (NT) DNA under similar conditions.

Glycerol gradients. Gradient solutions (v/v) are 20, 25, 30, 35, and 40% glycerol in 50 mM Tris-HCl, pH 7.5, stored at -20°C. Step gradients contained 1.0 ml 40% glycerol/50 mM Tris-HCl, pH 7.5, then 0.9 ml each concentration from 35-20%. Gradients were equilibrated 5-8 h at 4°C prior to use. Centrifugation was in a Beckman SW50.1 rotor at 48,000 rpm for 18 h at 4°C. Fractions were collected from the bottom of each tube using an Isco Gradient Fractionator and gravity flow. Fractions were routinely stored on ice, 4°C. AP endonuclease fractions were identified by activity on depurinated PM2 [^{3}H]DNA. The positions of molecular weight markers in the gradient were identified by their absorbance at 280 nm.

SDS-polyacrylamide gel electrophoresis. One-dimensional SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) using 6-11% acrylamide gels. Samples were

prepared by either dialysis vs. 1 mM Tris-HCl, pH 7.5, or by centrifugation in centricon-10 concentrators to remove any potassium phosphate and as well as to concentrate the samples. Molecular weight markers used for SDS-PAGE were phosphorylase b (90,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (29,000), trypsin inhibitor (20,000), and β-lactoglobulin (18,400). Gels were fixed and stained with Coomassie Brilliant Blue R, destained, and finally silver stained according to the method of Kelley (1984).

Dot blot analysis of nitrocellulose-bound proteins. Individual proteins were applied in 1 µl aliquots, with intermittent drying, to Millipore nitrocellulose membrane filters. The procedure of Burnette (1981) as described here, was followed. The filters were blocked by incubation at 37°C in a solution of 10 mM Tris-HCl, pH 7.4/0.9% NaCl/3% bovine serum albumin (Tris/Saline/BSA) for 1 h. The solution was then replaced with Tris/Saline/BSA containing a 1:50 dilution of rabbit antiserum prepared against HeLa AP endonuclease (Kane and Linn, 1981). Incubation was for 90 min at room temperature with gentle mixing. The antiserum was removed and the membrane rinsed with Tris/Saline for 10 min followed by two 20 min washes in Tris/Saline/0.005% NP-40. The membrane was then incubated for 30 min, with rocking, in a solution of Tris/Saline containing 4×10^7 cpm of [^{125}I]protein A from S. aureus (8×10^6 cpm/µg). The filter was then washed as described above. The membrane was blotted dry and exposed to Kodak XAR-5 film using a Dupont lightning-plus intensifying screen at -70°C.

Western blot. Duplicate SDS-PAGE gels were run to a maximum length of 19 cm. One gel was stained as described above; the second

gel was used for transfer of the proteins to nitrocellulose membranes using a Bio-Rad Transblot. Proteins were transferred as described in the Bio-Rad manual; 10 volts/0.1 A was applied for 3 h. The resulting nitrocellulose membrane was then blocked and probed as described above for Dot Blot analysis.

Other methods. The pH of buffers was routinely measured at 50 mM and room temperature. Protein determination followed the method of Bradford (1976) utilizing bovine serum albumin (BSA) as the standard. BSA was acetylated by the method of Dowhan (1969) with the acetylating agent acetic anhydride neutralized with 10% ammonium carbonate. Phosphocellulose and DEAE-cellulose were prepared by base washing prior to neutralization in appropriate buffers as described in RESULTS. Briefly, DNA agarose was essentially prepared according to Schaller et al. (1972). Calf thymus DNA (15 mg/ml) was stirred overnight in 20 mM NaOH, then denatured at 95°C for 15 min. This heat-denatured calf thymus DNA (HDCT) was then mixed thoroughly with 50 ml 4% agarose at 70°C. The HDCT-agarose was allowed to solidify on ice in a glass petrie dish, then forced through a metal sieve. The resultant slurry was washed extensively with 10 mM Tris-HCl, pH 7.5/ 1 mM EDTA/100 mM NaCl until the absorbance of the eluent at 260 nm was less than 0.1. The final DNA-agarose slurry was stored at 4°C in 10 mM Tris-HCl, pH 7.5/1 mM EDTA/1 M NaCl.

RESULTS

Separation of two apurinic/apyrimidinic endonucleases.

All purification steps were done at 4°C. All procedures utilized polyallomer tubes, when possible. The purification procedure of Spiering and Deutsch (1981) was followed with some modification.

Preparation of crude extract. Embryos (Oregon-R) were thawed by transferral from liquid nitrogen to -20°C for 30 min, then thawed completely on ice. Approximately 5 g embryos were used in each extraction. Embryos were transferred to a ground glass homogenizer using 0.5-1.0 ml Buffer A (50 mM potassium phosphate, pH 7.5/1 mM EDTA and 25 µl Aprotinin per ml buffer) per gram of embryos. Following homogenization, the suspension was centrifuged at 27,000 x g for 30 min. The supernatant was set aside on ice, while the pellet was resuspended in a minimal volume of Buffer A, sonicated with 4 x 30 s bursts with a Biosonic sonicator (needle probe, 3 amps) and then centrifuged at 12,000 x g for 15 min. The combined supernatants were then filtered through sterile HD 100 Nitex mesh to remove lipid-like material and brought to 0.4 M NaCl with the addition of 5 M NaCl. Endogenous DNA was removed by the addition of fresh, sterile 6.0% streptomycin sulfate, pH 7.0, to a final concentration of 0.6%, stirred for 30 min on ice and then centrifuged at 27,000 x g for 30 min. The resulting supernatant was filtered through sterile HD 100 Nitex mesh and dialyzed 3 h against 1 liter 25 mM potassium phosphate. pH 7.5/0.2 mM EDTA/5% glycerol/10 mM β-mercaptoethanol (25 mM KE

Buffer). Aprotinin, 10 µl per ml buffer, was added to the post-dialysate to account for any Aprotinin lost during dialysis. Centrifugation at 12,000 x g for 15 min removed any debris resulting from dialysis.

Phosphocellulose chromatography. A phosphocellulose column (115 mg extract/20-ml phosphocellulose) was equilibrated with 50 mM KE Buffer plus 25 µl Aprotinin per ml buffer (50 mM KEA Buffer). The post-dialysate extract was applied to the column at a flow rate of 0.3 ml/min. Following sample application, the column was washed with 2-3 column volumes of 50 mM KEA, eluting a major protein peak detectable by UV monitoring at 280 nm. The shoulder of this protein peak contained AP endonuclease I. A 100 mM KEA Buffer wash was then applied to remove a second protein peak prior to gradient application. A 100-600 mM KEA Buffer gradient (4 x column volume) was then applied, followed by a 600 mM KEA Buffer wash. One-ml fractions were collected in which alternate fractions throughout the column were assayed for AP endonuclease activity. AP endonuclease II eluted between 200 and 400 mM potassium phosphate. A typical endonuclease profile is shown in Figure 1. The column flow-through and 100 mM KEA Buffer-wash fractions were stabilized with the addition of 10 mg/ml acetylated BSA (final concentration of 100 µg/ml) and 0.5% Triton X-100 (final concentration of 0.005%). The gradient and high-salt wash fractions received only the Triton X-100 addition. Flow-through fractions containing peak AP endonuclease activity were pooled as AP endonuclease I, Fraction II. High-salt fractions containing AP endonuclease activity were combined as AP endonuclease II, Fraction II.

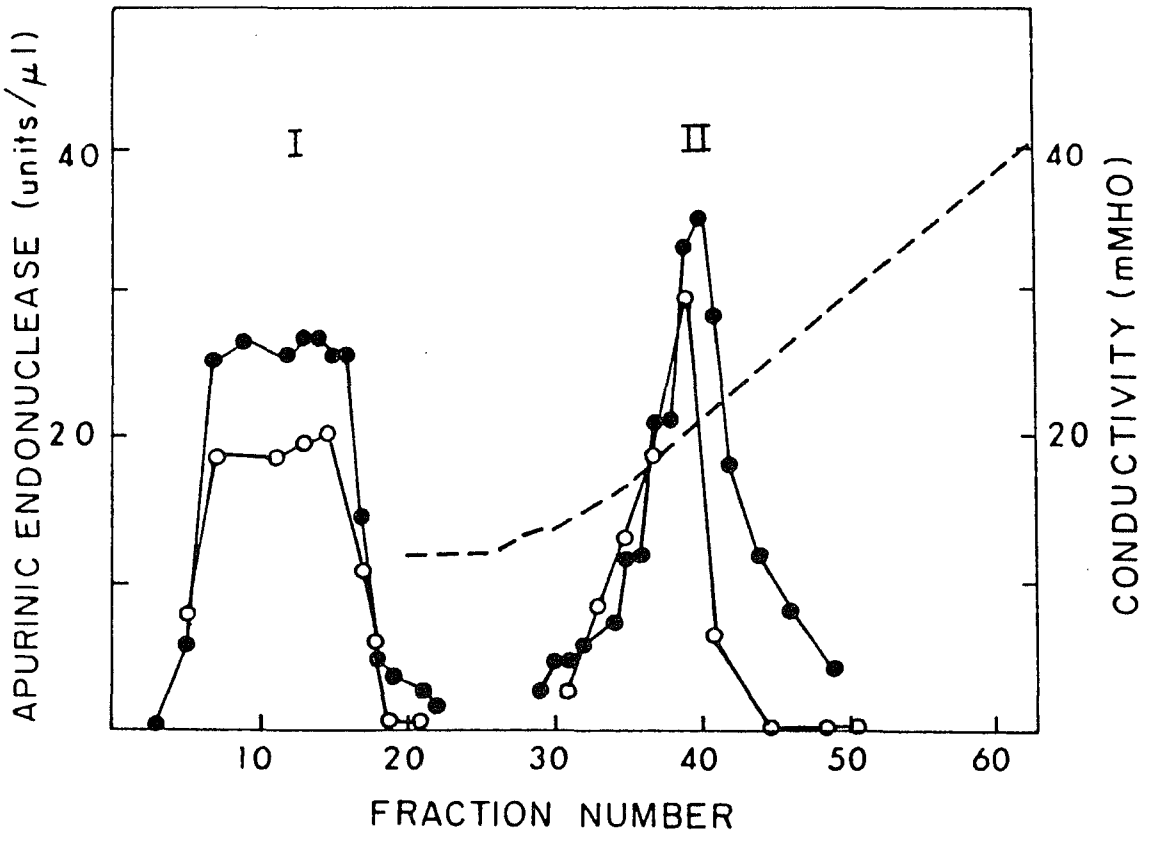


Figure 1. Phosphocellulose chromatography.

Reactions were as described in Materials and Methods. AP endonuclease I is the column flow-through, while AP endonuclease II elutes at 200-400 mM potassium phosphate. (●-●) Activity on depurinated DNA; (o-o) activity on non-treated DNA.

Further Purification of AP Endonuclease I

DEAE-cellulose chromatography. The procedure of Spiering and Deutsch (1981) was modified as follows. DEAE-cellulose (5-ml) was poured in a plastic syringe. The column was equilibrated with 50 mM KCl/20 mM Tris-HCl, pH 7.5/10 mM β-mercaptoethanol/0.2 mM EDTA/5% glycerol and 25 µl Aprotinin per ml buffer. AP endonuclease I activity eluted in the column flow-through; these fractions also contained some non-specific nuclease activity (Fig. 2). Peak fractions were pooled as AP endonuclease I, Fraction III.

Affi-Gel blue chromatography. The procedure of Kane and Linn (1981) was essentially followed with the following modifications. A 3-ml Affi-Gel blue column was equilibrated in 50 mM Tris-HCl, pH 7.5/0.1 mM EDTA/0.05% Triton X-100 and 25 µl Aprotinin per ml buffer. The pooled DEAE-cellulose activity (Fraction III) was applied to the column at a flow rate of 0.2 ml/min and the column flow-through collected as fraction 1. The column was washed with 3 x column volumes of equilibration buffer, collected as fraction 2. This was followed by 6 ml of equilibration buffer containing 1 mM NAD^+/5 mM ATP. This was collected in one tube as fraction 3. The NAD^+/ATP solution was washed from the column with equilibration buffer and collected in a single tube as fraction 4. Next, a 0-0.5 M KCl gradient was applied to the column and one-ml fractions collected. Individual column fractions were assayed for AP endonuclease activity at dilutions sufficient to remove the inhibitory effects of NAD^+/ATP (Fig. 3). AP endonuclease I activity eluted from the Affi-Gel blue in

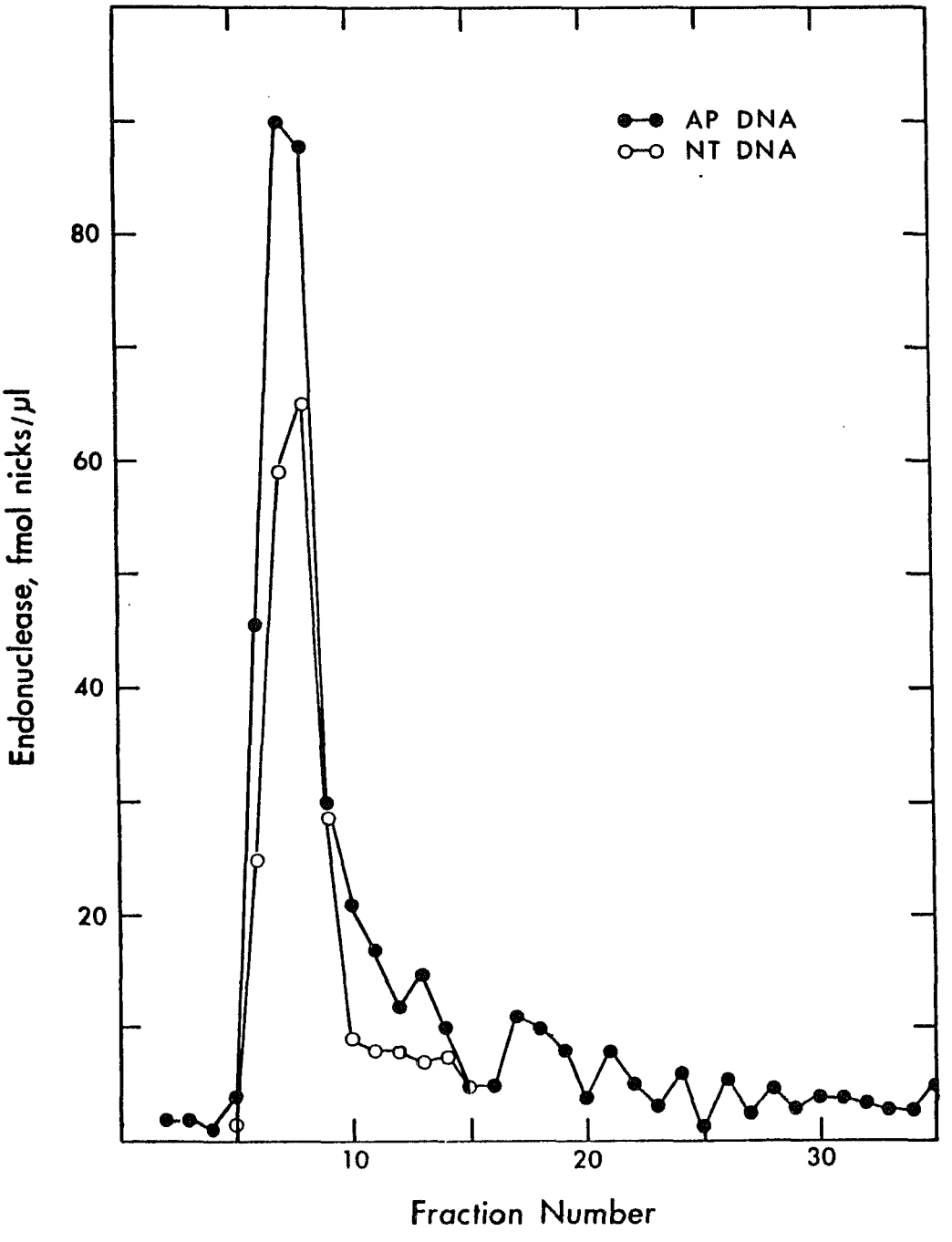


Figure 2. DEAE-cellulose chromatography of AP endonuclease I.

Phosphocellulose fractions containing activity towards depurinated DNA were pooled and applied to a DEAE-cellulose column as described in the text.

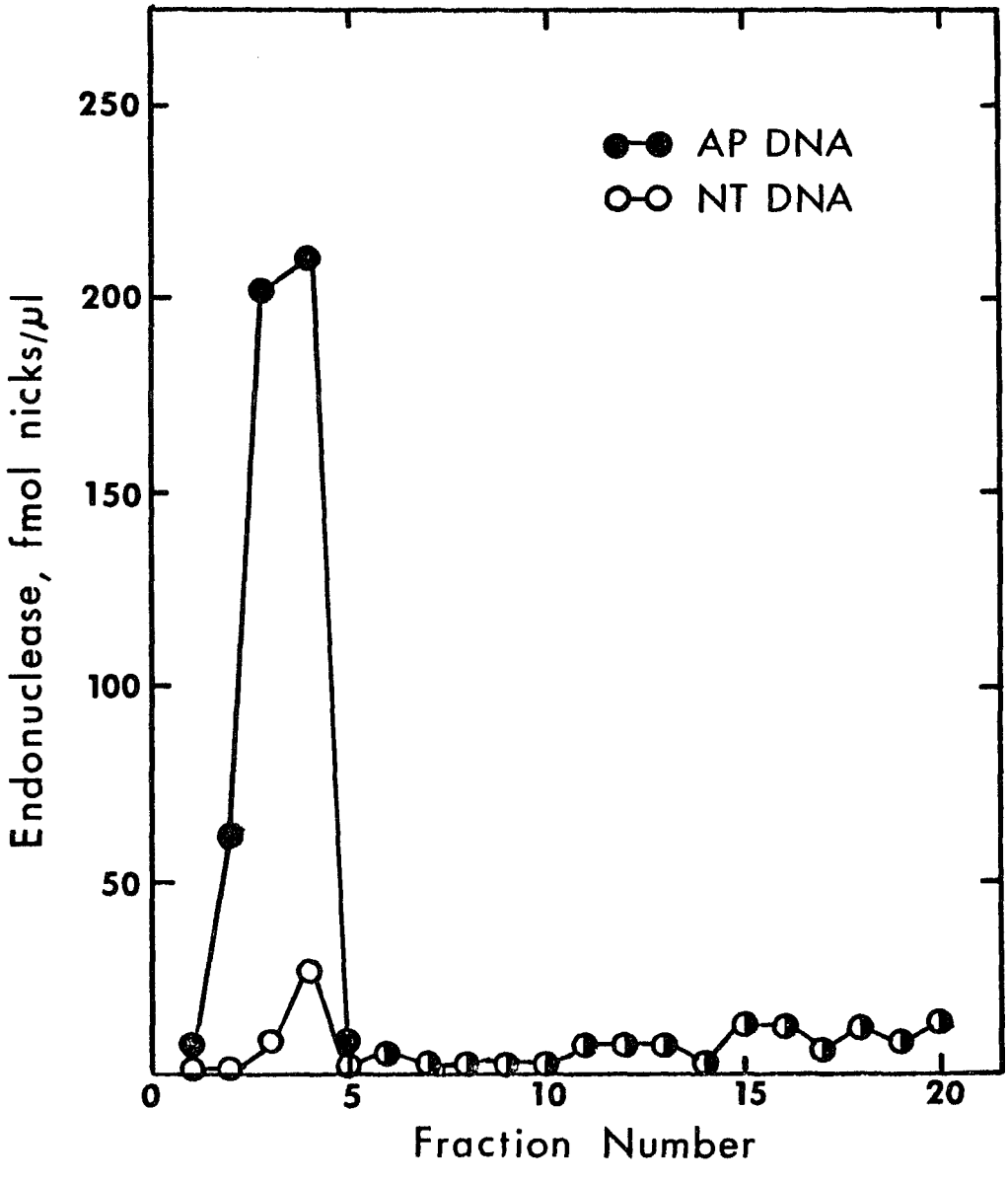


Figure 3. Affi-Gel blue chromatography of AP endonuclease I.

Fractions were assayed for AP endonuclease activity as described in Materials and Methods.

the NAD^+/ATP wash. Peak fractions were pooled as AP endonuclease I, Fraction IV. The purification scheme is summarized in Table I.

Further Purification of AP Endonuclease II

DNA-agarose chromatography. A 3 x 1.5 cm DNA-agarose column was poured in a plastic syringe and equilibrated with 20 mM Tris-HCl, pH 7.5/0.2 mM EDTA/5% glycerol/10 mM β-mercaptoethanol (TEGM Buffer). AP endonuclease II, fraction II, was prepared for DNA-agarose chromatography by dialysis against TEGM Buffer for 24 h, 4°C. Fraction II was applied to the column at a flow rate of 0.2 ml/min; one-ml fractions were collected. Following sample application, a 40-ml TEGM Buffer wash was applied to remove any protein not bound to the DNA-agarose matrix. A 0-600 mM NaCl gradient in TEGM Buffer was then applied. AP endonuclease II eluted in a broad peak between 150 and 450 mM NaCl (Fig. 4). Fractions containing AP endonuclease activity were pooled as AP endonuclease II, Fraction III and brought to 0.005% Triton X-100 prior to storage on ice at 4°C.

Sample concentration. Due to the broad activity peak obtained from DNA-agarose chromatography, concentration was necessary to reduce the sample volume. The use of centricon-10 concentrators, with centrifugation at 5500 rpm in a Beckman JA-20 rotor, resulted in a 22-fold concentration of AP endonuclease II with no loss of activity.

Sephadex G-100 chromatography. A 46 x 2.5 cm column of Sephadex G-100 was equilibrated with 100 mM potassium phosphate, pH 7.5/0.2 mM EDTA/5% glycerol/10 mM β-mercaptoethanol/0.005% Triton X-100. The

Table I. Purification of AP endonuclease I.

Fraction	Volume	Total units[a]	Total protein	Specific activity
	ml		mg	units/mg protein
I. Crude	24.9	2913	202	14
II. Phosphocellulose	13.8	97	10	10
III. DEAE-cellulose	3.7	475	4	119
IV. Affi-Gel blue	15.9	374	<0.1	4990

[a]This value has been corrected for the amount of activity detected toward nontreated DNA.

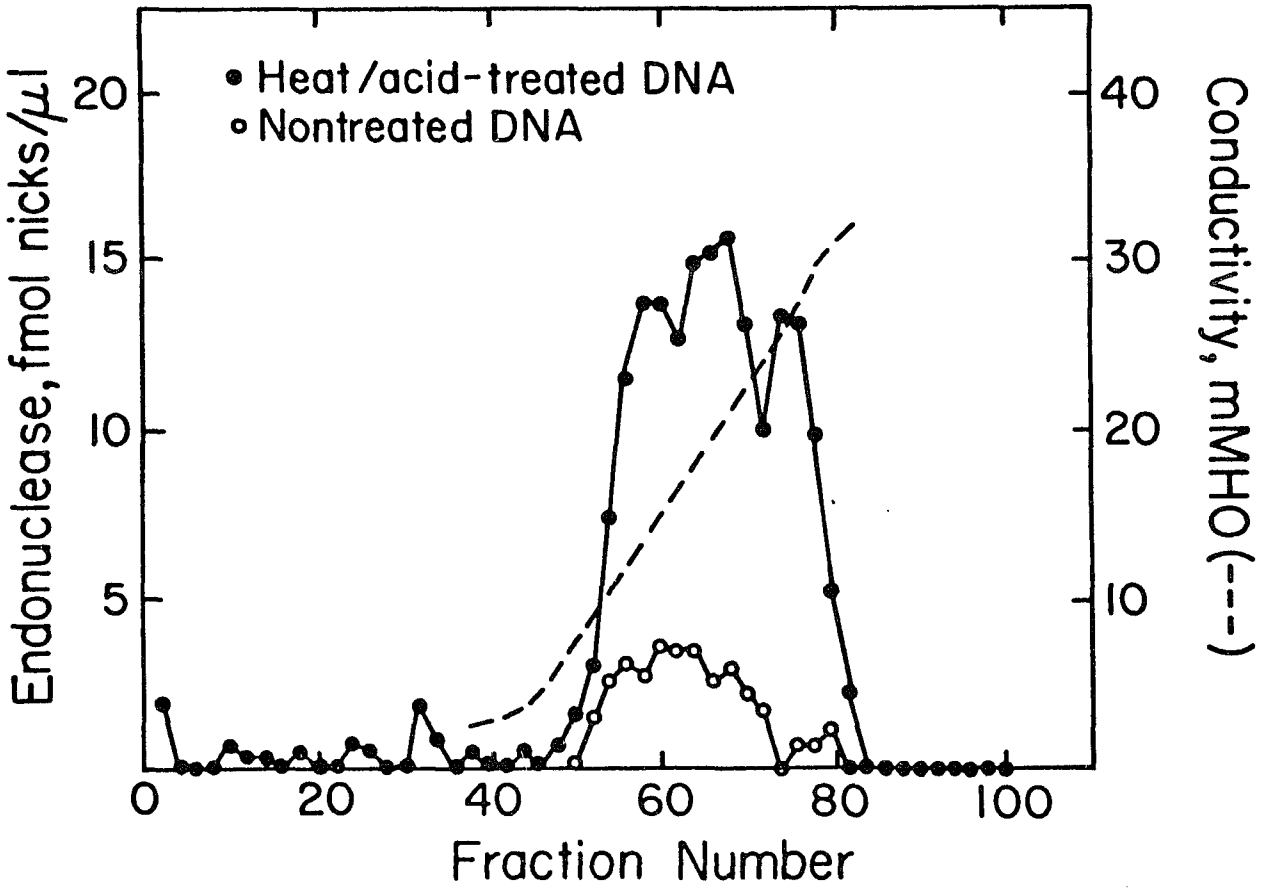


Figure 4. DNA-agarose chromatography of AP endonuclease II.

AP endonuclease II, Fraction II, was applied to a DNA-agarose column as described in the text. Fractions throughout the column were assayed for specific nuclease activity on depurinated DNA as described in Materials and Methods.

column was calibrated with blue dextran 2000 (V_o), bovine serum albumin (67,000), ovalbumin (43,000), β-lactoglobulin (36,800 and 18,400), carbonic anhydrase (29,000), cytochrome c (12,400), and phenol red (V_i) at a flow rate of 0.6 ml/min. Following application of 0.5 ml AP endonuclease II, Fraction IV, one-ml fractions were collected under identical conditions used for column calibration. Two major protein peaks eluted from the column -- one in the void volume and a second peak at an elution volume corresponding to 10,000 daltons. Neither of these protein peaks contained any nuclease activity. A sharp peak of AP endonuclease II activity eluted following the V_o (Fig. 5). A plot of the log molecular weights of the protein standards vs. their elution volumes was used to calculate a molecular weight of 67,000 for AP endonuclease II by linear regression. Non-specific endonuclease activity eluted in a broad peak corresponding to 44,000. The purification scheme is summarized in Table II.

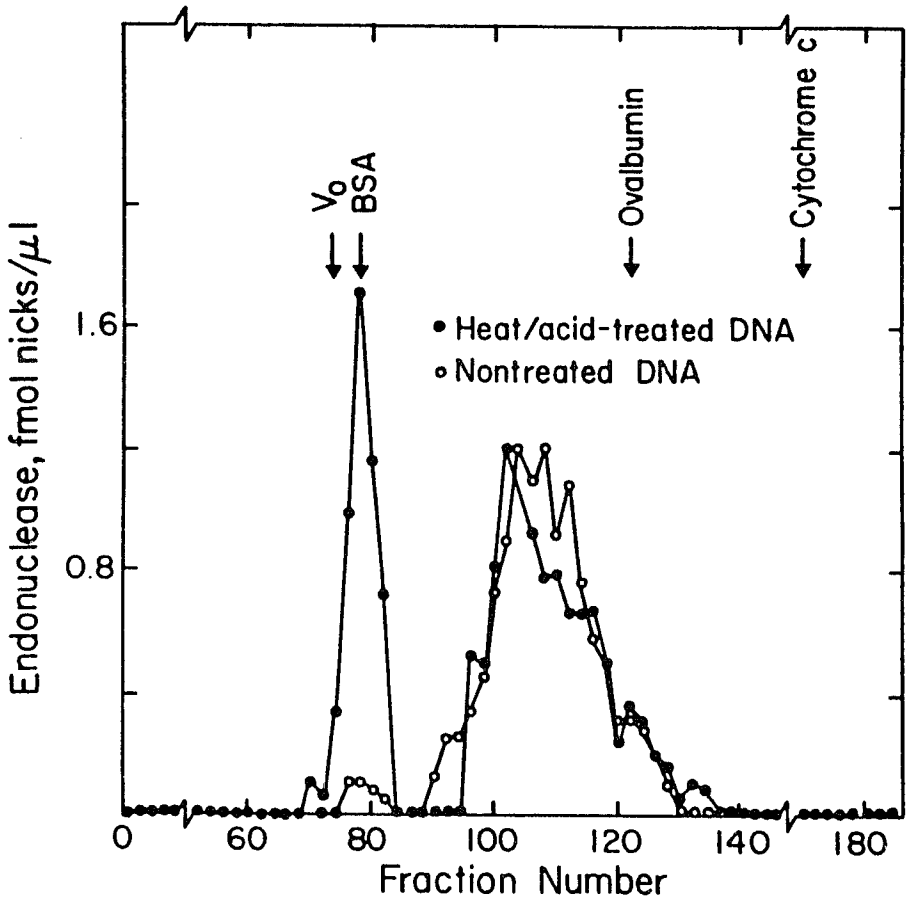


Figure 5. Sephadex G-100 chromatography of AP endonuclease II.

AP endonuclease II, Fraction IV, was applied to a Sephadex G-100 column as described in the text. Fractions preceding the V_o through a molecular weight corresponding to 10,000 were assayed for AP endonuclease activity. Elution of proteins for molecular weight analysis was under identical conditions.

Table II. Purification of AP endonuclease II.

Fraction		Volume	Total units[a]	Total protein	Specific activity
		ml		mg	units/mg protein
I.	Crude	48	11712	1520	8
II.	Phosphocellulose	30	660	6	110
III.	DNA-agarose	40	304	0.5	608
IV.	DNA-agarose concentrate	2	289	0.4	722
V.	Sephadex G-100[b]	8	30	<0.1	≥3000

[a]This value has been corrected for the amount of activity detected toward nontreated DNA.

[b]Fraction V resulted from the application of approximately 25% Fraction IV activity (0.5 ml) to a Sephadex G-100 column as described in the text.

Characterization of Enzymes

Comparison of enzyme activities from two strains of D. melanogaster. To ascertain that the two species of AP endonuclease isolated from Oregon-R embryos were representative of the wild-type condition, parallel extractions in Canton-S and Oregon-R strains were undertaken. Embryos (8 h) were homogenized and the AP endonucleases purified through the phosphocellulose chromatography step as described above. Comparable activities of AP endonuclease I and II were obtained from the Canton-S embryos upon comparison to Oregon-R embryos (Table III). As Oregon-R stocks were in a greater abundance than Canton-S stocks, Oregon-R embryos were used in all subsequent characterization studies.

pH optimum. The two D. melanogaster AP endonucleases exhibit different responses to changes in pH. AP endonuclease I has a very sharp optimal pH of 6.5. AP endonuclease II has a pH optimum of 7.0, with retention of a greater percentage of activity at basic pH's than observed for AP endonuclease I (Fig. 6).

Reaction requirements. One method of differentiating between AP endonuclease I and II is by their response to divalent cations (Table IV). AP endonuclease II activity is undetectable in the absence of any added divalent cations, with 10 mM $MgCl_2$ giving optimum activity. $MnCl_2$ (10 mM) partially substitutes for $MgCl_2$. Conversely, the addition of any divalent cations has no effect upon the apurinic activity of AP endonuclease I, but does result in an increase in its associated non-specific nuclease. The addition of EDTA to the

Table III. Comparision of AP endonuclease activities from two wild-type D. melanogaster strains.

Fraction		Specific Activity (units/mg)[a]	
		Canton-S	Oregon-R
I.	Crude	12-16	8-12
II.	Phosphocellulose AP endonuclease I	14	30
	AP endonuclease II	125	110

[a]This value has been corrected for the amount of activity detected toward nontreated DNA.

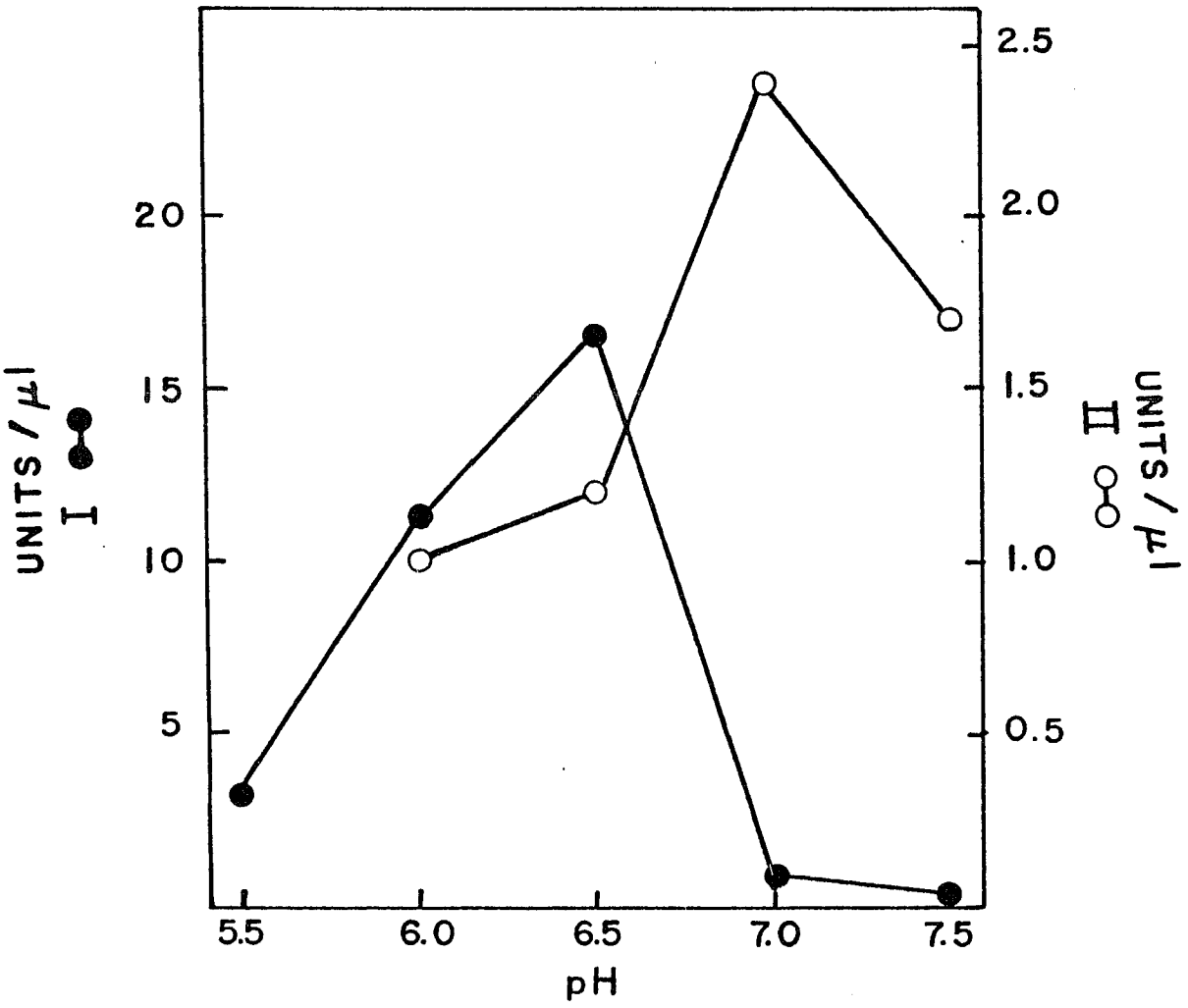


Figure 6. pH optima.

Reactions were as described in Materials and Methods. Reaction mixtures contained 25 mM Tris-HCl and approximately 50 fmol PM2 [^{3}H]DNA molecules. AP endonuclease II reactions also contained 10 mM $MgCl_2$.

Table IV. Reaction requirements.
Reaction mixtures (50 µl) contained 25 mM Tris-HCl, pH 6.5 (for AP endonuclease I, 0.02 unit enzyme) or pH 7.0 (for AP endonuclease II, 0.02 unit enzyme) and 40-50 fmol PM2 [^{3}H]DNA, 2 AP sites/genome. Reaction conditions were as described for nuclease reactions in Materials and Methods.

Additions	AP Endonuclease I AP	I NT	II AP	II NT
	% Relative Activity			
None	100	100	<1	<1
+ $MgCl_2$				
0 mM	100		<1	<1
2			28	
5			54	
10	100	153	100	100
+ $MnCl_2$				
2 mM			14	
5			40	
10			69	
+ $CaCl_2$				
4 mM	100	278	30	<1
10			54	120
25			<1	<1
+ KCl[a]				
1 mM			102	60
5			97	80
10	185	156	114	80
20			130	80
50			181	180
100			81	160
+ NaCl, 10 mM	156	144		
+ EDTA[a]				
1 mM	105	100		
10			<1	<1
+ EGTA[a]				
1 mM	124	162		
5			118	
10			136	

[a]AP endonuclease II reactions also contained 10 mM $MgCl_2$.

reaction mixtures inhibits AP endonuclease II activity with no effect on AP endonuclease I activity (Table IV). This correlates well with the lack of stimulation of AP endonuclease I by any known divalent cations. EGTA does not affect the apurinic endonuclease activity of either AP endonuclease. The addition of KCl has little effect on AP endonuclease II, while stimulating the non-specific nuclease associated with AP endonuclease I. The addition of either $CaCl_2$, KCl, or NaCl stimulates the non-specific nuclease associated with AP endonuclease I; any increase in AP activity is countered by an increase in non-specific activity. Conversely, the presence of $CaCl_2$ in AP endonuclease II reactions inhibits both specific and non-specific nuclease activities.

Effects of other small molecules. Harmane inhibits both enzymes at a concentration of 1 mM (Table V). It has been theorized that harmane is inhibitory only to those AP endonucleases with associated DNA glycosylase activity (Warner et al., 1981). Additionally, the presence of NAD^+ or ATP inhibits both AP endonuclease activities. Adenine or dNTPs also inhibit AP endonuclease II. The purine analog caffeine, however, appears to have no effect on AP endonuclease II activity.

Enzyme specificity. The two enzyme species were monitored during purification by assaying for specific apurinic nuclease activity. To ensure that the nicking assay allows quantitation of specific AP endonuclease activity, PM2 [^{3}H]DNA was depurinated 6.5 min to yield approximately 1.2 AP sites per genome. The phosphocellulose fractions of AP endonucleases I and II were incubated with this substrate for various lengths of time. The reaction mixtures were then divided and

Table V. Effects of small molecules.
Reaction mixtures (50 µl) contained 25 mM Tris-HCl at pH 6.5 (AP endonuclease I, 0.2 units) or pH 7.0 and 10 mM $MgCl_2$ (AP endonuclease II, 0.2 units). PM2 [^{3}H]DNA was depurinated to contain 1.2 AP sites/genome. Reaction conditions were as described in Materials and Methods, using 250 fmol DNA molecules.

Addition	AP Endonuclease I	AP Endonuclease II
	% Relative activity	
None	100	100
+ Caffeine		
1 mM		54
5		106
10		98
+ Harmane		
0.5 mM		27
1.0	<1	30
2.0		<1
+ NAD		
1.0 mM		39
5.0	20	6
+ ATP		
1.0 mM		13
5.0	16	5
+ Adenine		
0.1 mM		73
0.2		64
+ dCTP		
1 mM		16
4		5
+ dGTP		
1 mM		52
4		8
+ TTP		
1 mM		44
4		9

one-half tested for AP endonuclease activity as described in MATERIALS AND METHODS. The remaining reaction mixture was treated at pH 12.3 for 4 h at 25°C. Apurinic sites are alkali-labile and should be hydrolyzed by this treatment. As shown in Figure 7, as time increases, both the endonuclease I and endonuclease II reactions approach 1.2 nicks/DNA molecule, the approximate maximum AP sites/genome. AP endonuclease I exceeds the 1.2 nicks/genome limit slightly; this may be due to non-specific nuclease activity.

Other DNA substrates. The two endonucleases were purified by assaying for endonuclease activity on apurinic PM2 [^{3}H]DNA, though apurinic sites are only one of a multitude of damaged sites seen in DNA. To test for enzymatic activity on apyrimidinic DNA, PM2 [^{3}H]DNA was treated with sodium bisulfite to introduce approximately 1 dUMP residue per genome (Lindahl et al., 1977), and then incubated with E. coli uracil DNA-glycosylase to yield approximately 1.3 apyrimidinic sites per genome (Green and Deutsch, 1984). Apyrimidinic DNA was then incubated with enzyme and the nicks compared with enzymatic activity on apurinic DNA. The results of this reaction are seen in Table VI. Both AP endonucleases I and II react with either apyrimidinic or apurinic DNA, given the same number of baseless sites. Even though the observed activity is less for apyrimidinic DNA, the difference is considered to be insignificant.

To determine if either Drosophila AP endonuclease was reactive toward alkylated DNA, unlabelled PM2 DNA was treated with methyl methanesulfonate (MMS) to contain 4 alkali-labile sites/DNA molecule. This treatment should result in a DNA substrate primarily methylated at N^{7}guanine, N^{3}adenine, and N^{1}adenine (although other lesions do

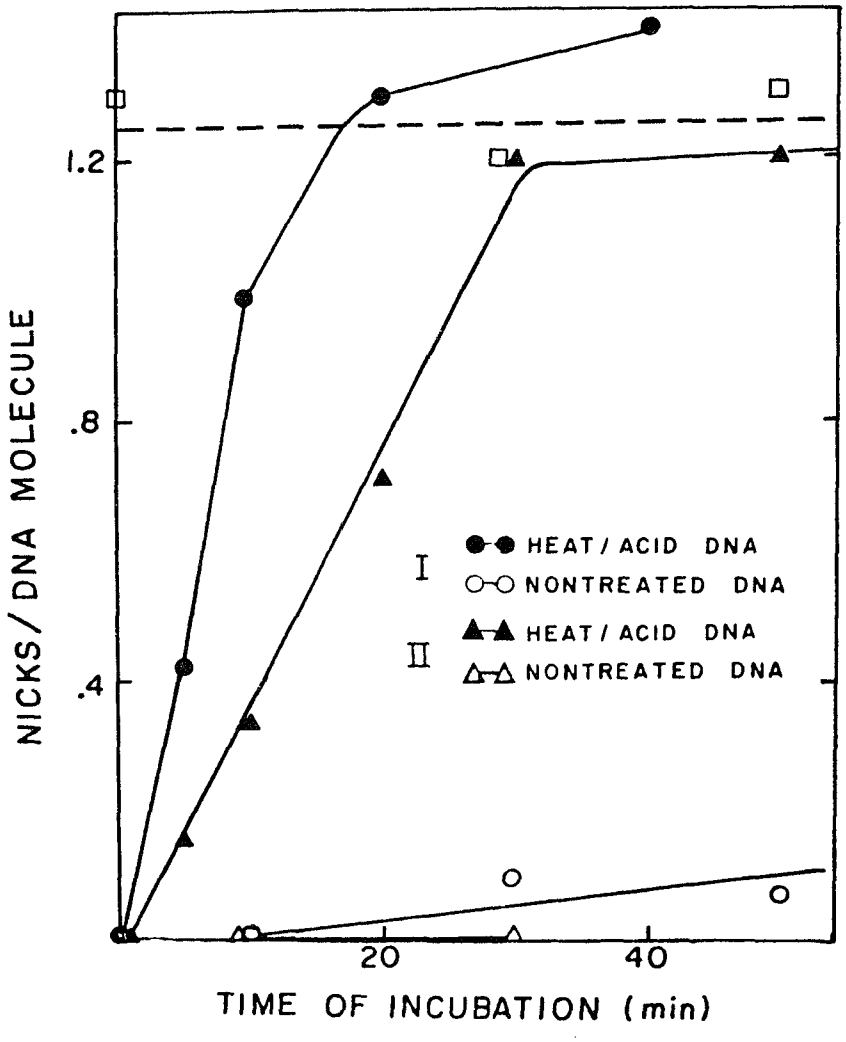


Figure 7. Specificity of the endonuclease activities.

Assays were as described in the text with 9.2 units/µl AP endonuclease I and 3 units/µl AP endonuclease II. (□-□) Alkali-labile sites plus enzyme; (- -) Alkali-labile sites minus enzyme.

Table VI. Endonuclease activity on apurinic vs. apyrimidinic DNA.

DNA substrates and reaction conditions were as described in the text. Both substrates contain approximately 1.2 AP sites for endonuclease cleavage.

Substrate	AP Endonuclease	
	I	II
	Units	
Apurinic PM2 [^{3}H]DNA	48	65
Apyrimidinic PM2 [^{3}H]DNA	37	48

exist, such as modified pyrimidines). The standard nuclease reaction mixture containing depurinated PM2 [^{3}H]DNA was augmented with MMS-treated PM2 DNA to determine if this unlabelled DNA substrate would act as a competitive substrate for the AP DNA. Activity toward alkylated DNA would, therefore, be evidenced by a decrease in activity on the AP [^{3}H]DNA. (Nuclease activity on MMS-treated DNA could not be determined directly by the standard filter-binding nuclease assay as alkylated DNA binds to the nitrocellulose filters with or without incision.) Table VII shows the results of this complementation assay. MMS-treated PM2 DNA is not a substrate for either of the Drosophila AP endonucleases, however, if the MMS-treated DNA is heated to either 50°C or 70°C to create AP sites, some activity is observed. This is due to release of the methylated bases, resulting in the presence of apurinic sites which are then recognized by the D. melanogaster enzymes.

Osmium tetroxide (OsO_4)-treated PM2 DNA was also tested as a substrate for endonuclease activity. This treatment mimics the effects of free radical formation in DNA by the production of thymine glycols. Both D. melanogaster AP endonucleases are unreactive toward this substrate.

Additionally, PM2 [^{3}H]DNA was heat-denatured at neutral pH for 5 min at 100° C and tested as a substrate for the two AP endonucleases. Neither enzyme exhibited any activity on this single-stranded DNA above the activity seen on nontreated DNA.

To determine if the apurinic/apyrimidinic endonucleases were reactive toward thymine dimers, PM2 [^{3}H]DNA was UV-irradiated to an extent that would produce approximately 2 thymine dimers per PM2

Table VII. Activity of AP endonucleases I and II on alternate DNA substrates.

Reaction conditions were as described in Results. Adapted from Spiering and Deutsch (1981).

Addition to DP PM2 [^{3}H]DNA	AP Endonuclease I	AP Endonuclease II
	% Relative Activity	
None	100	100
MMS-treated PM2 DNA	100	67
MMS-treated PM2 DNA, heated to 50°C	<20	<20
Non-treated PM2 DNA	100	100

genome at the highest exposure used (Deutsch et al., 1976). Enzymes were used at a concentration to nick approximately 1 AP site/genome. There was no activity on UV-treated DNA substrate by either of the AP endonucleases above that seen on non-treated DNA (Table VIII).

Stability of the enzymes. AP endonuclease I is the more labile of the two enzymes, losing as much as 50% activity seven days after phosphocellulose chromatography. AP endonuclease II is stable for short periods of time at 4°C, but loses approximately 80% of the activity within 30 days at 4°C. To enhance the stability of both enzymes, a variety of storage buffers were analyzed (Table IX). The routine addition of 0.005% Triton X-100 and 100 µg/ml acetylated BSA to AP endonuclease I and 0.005% Triton X-100 to AP endonuclease II extracts resulted from this analysis.

Neither enzyme was stable to storage at -20°C without Triton X-100 addition. With the addition of Triton X-100 to AP endonuclease II, the enzyme could be frozen at -20°C and thawed with very little loss of activity. Repeated freezing and thawing of the enzyme did show a marked decrease in activity as compared to samples which had not been previously thawed. AP endonuclease I, however, is a much more labile enzyme. Even with the addition of Triton X-100 and acetylated BSA, AP endonuclease I could not be stored at -20°C with extensive retention of enzymatic activity. Within three days, activity dropped by 50%. The addition of glycerol did not appear to increase the stability of either AP endonuclease or of the non-specific nuclease associated with AP endonuclease I. Due to their instability over long periods of time at -20°C, both AP endonuclease I and II were routinely stored on ice at 4°C.

Table VIII. UV-treated PM2 [^{3}H]DNA as a substrate for AP endonucleases I and II.

Reactions were as described in the text with 1 x 10^3 units AP endonuclease I and 5 x 10^3 units AP endonuclease II as determined by activity on depurinated DNA.

UV Treatment	AP Endonuclease	
	I	II
	Activity, Units	
0 s	60	33
30 s	81	79
60 s	58	33
180 s	72	60

Table IX. Storage conditions for D. melanogaster AP endonucleases. Phosphocellulose fractions were stored as indicated. Relative activity was determined by the nuclease assay described in Materials and Methods, using 0.3 unit of enzyme.

Storage conditions	AP Endonuclease I	AP Endonuclease II
	% Relative Activity	
4°C		
no additions	100	100
4 days		81
65 days	0	18
Acetylated BSA, 100 µg/ml	78	100
Triton X-100, 0.005%	100	
0.01%	80	
Glycerol, 50%	42	
-20°C, 3 days		
no additions	0	73
Acetylated BSA, 100 µg/ml	15	61
Triton X-100, 0.005%	17	80
0.01%	23	0
Glycerol, 50%		
3 days	0	61
4 days (re-thawed)		13

Associated/contaminating activities. AP endonuclease I contains a non-specific nuclease which consistently co-purifies with the AP endonuclease activity, present in the ratio of 60% AP to 40% NT nuclease activity when saturating enzyme concentrations were used in the nuclease assay. Attempts to separate the two nucleases chromatographically have been completely unsuccessful. For example, hydroxylapatite or single-stranded DNA-cellulose chromatography does not show improved separation of the two activities. Additionally, the non-specific nuclease shows no activity on any other DNA tested to date -- MMS, OsO_4, UV, or heat-denatured DNA. Conversely, AP endonuclease II may be purified away from non-specific nuclease activity (see Fig. 5). Both endonucleases appear to lack exonuclease and phosphomonoesterase activities under conditions employed fpr detecting AP endonuclease activity using a variety of DNA substrates. AP endonuclease II has slight phosphomonoesterase activity present in the phosphocellulose fractions as detected by the loss of ^{32}P on [dAMP-5'-^{32}P, uracil-^{3}H]poly(dA-dT) DNA substrates. This activity, however, is not present in more purified fractions.

Glycerol gradient analysis. Glycerol gradients (20-40%) were run for two purposes: to determine the sedimentation coefficient for the two AP endonucleases and as a purification step to separate any contaminating activities from the desired endonucleases. Figure 8 shows a typical glycerol gradient profile for AP endonucleases I and II. Separation of both an apurinic DNA binding activity and a non-specific nuclease activity from AP endonuclease II, Fraction II, is possible by glycerol gradient analysis. Unfortunately, AP endonuclease I could not be separated from contaminating non-specific

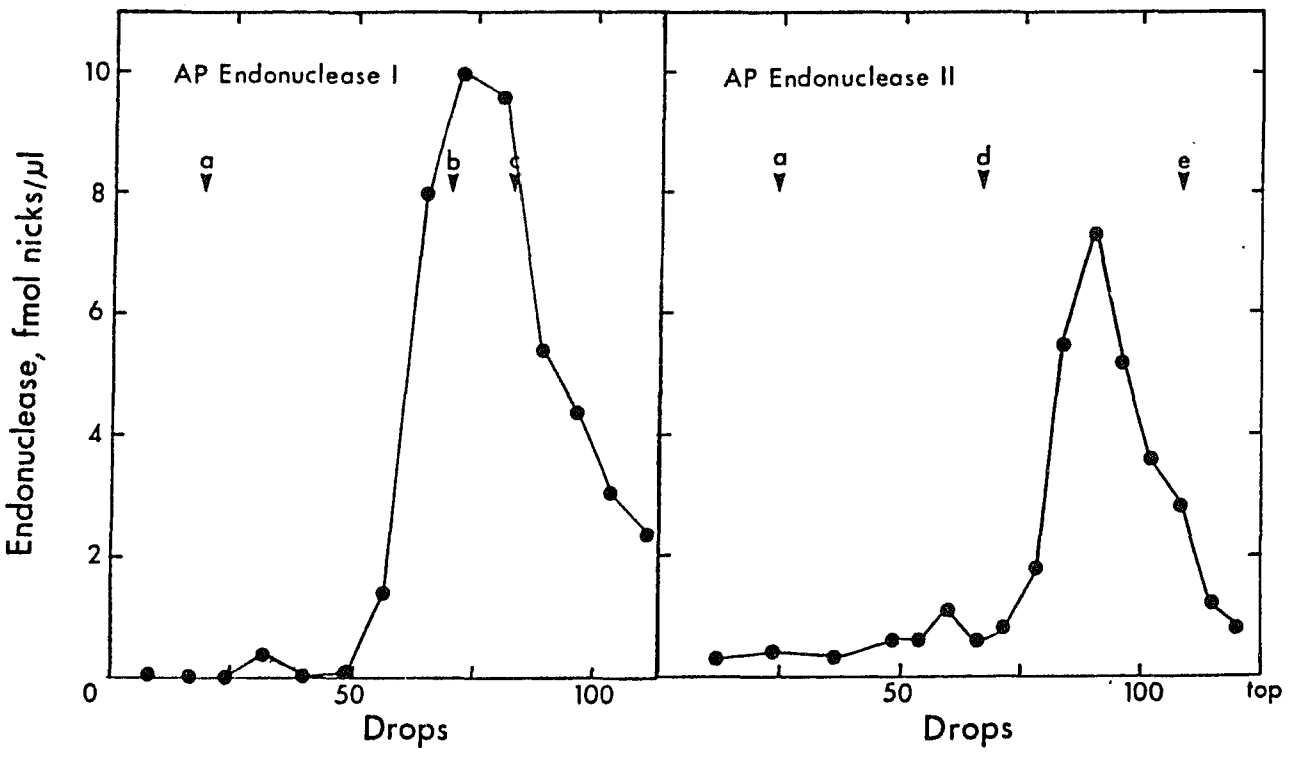


Figure 8. Glycerol gradient analysis of AP endonucleases I and II.

Glycerol gradients were run as described in Materials and Methods with determination of AP endonuclease activity by the standard nuclease assay. Molecular weight markers used were: a, bovine serum albumin (67,000); b and d, ovalbumin (43,000); c, β-lactoglobulin (36,800); e, lactalbumin (14,400).

nuclease activity by this method. The relative s values for both enzymes were calculated from data in which the molecular weight markers migrated consistent with their known sedimentataion coefficients. By linear regression analysis (r=0.95), AP endonucleases I and II were calculated to have relative s values of 2.5S and 2.8S, respectively. Using the method of Ackers (1964) and known Stokes radii for the molecular weight markers used, the Stokes radius for AP endonuclease II on Sephadex G-100 was calculated to be 38Å, assuming a partial specific volume of 0.725 cm^2 g^{-1}. From the Stokes radius and the corresponding s value, an approximate molecular weight could be calculated based upon the method of Siegel and Monty (1966). AP endonuclease II is calculated to have a molecular weight of 39,189. Unfortunately, the lability of AP endonuclease I on Sephadex G-100 precluded conducting similar calculations.

SDS-PAGE analysis. D. melanogaster AP endonuclease I is not purified to homogeneity after Affi-Gel blue chromatography, as several bands are still present on silver stained SDS-PAGE (Fig. 9). AP endonuclease II may be purified to homogeneity following Sephadex chromatography, but the protein concentration of the resulting enzyme fractions is below the detection of silver staining (i.e. less than 5 ng protein). Unfortunately, further concentration of AP endonuclease II does not allow for the visualization of any protein using Coomassie Blue or silver staining.

Dot blot analysis. Since it was impossible to determine the precise molecular weight of either of the AP endonucleases by visualization on gels, an attempt was made to cross-react the D. melanogaster enzymes with a polyclonal antibody to a human Class II

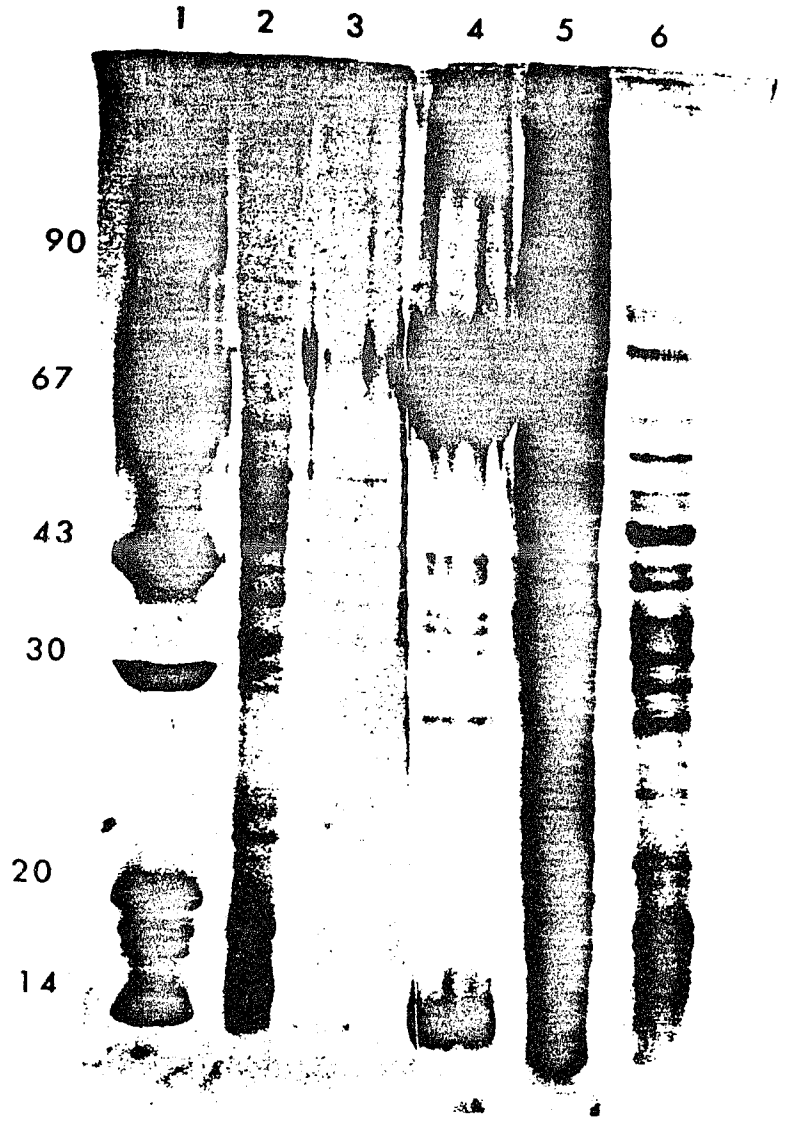


Figure 9. SDS-PAGE analysis of purification steps.

Samples run on the gel were: 1, molecular weight markers (see Materials and Methods); 2, AP endonuclease II, Fraction III, 15 µg; 3, AP endonuclease II, Fraction II, 10 µg; 4, AP endonuclease I, Fraction IV, 80 µg; 5, AP endonuclease I, Fraction II, 31 µg; 6, AP endonucleases I and II, Fraction I, 20 µg. SDS-PAGE were prepared according to the procedure of Laemmli (1960) and run for 3 h at 20 watts.

AP endonuclease, HeLa AP endonuclease (Kane and Linn, 1981). If the D. melanogaster enzymes would cross-react, Western analysis could be done to precisely locate the specific proteins from a SDS-PAGE. Both AP endonucleases I and II were found to cross-react with the antibody (Fig. 10). Additionally, the antibody was found to cross-react with a prokaryotic Class II AP endonuclease, E. coli endonuclease IV, but not with another D. melanogaster enzyme, dUTPase.

Western blot analysis. Using the standard SDS-PAGE and Western blot techniques, the results shown in Figure 11 were obtained. By comparison to molecular weight standards run simultaneously, the antibody cross-reacts with a single 68,000 dalton protein for AP endonuclease I (lane A) and with a single 64,000 dalton protein for AP endonuclease II (lane B).

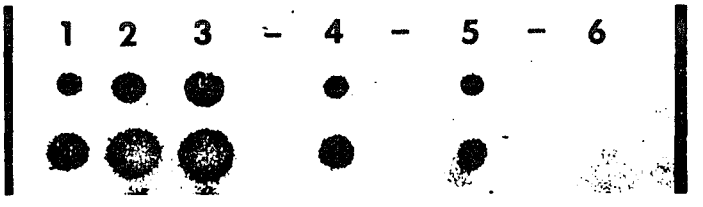


Figure 10. Dot blot analysis.

Proteins applied to a nitrocellulose filter were: 1, HeLa AP endonuclease (10 ng/µl); 2, Crude extract (20 ng/µl); 3, AP endonuclease I, fraction IV (14 ng/µl); 4, AP endonuclease II, fraction IV (750 ng/µl); 5, *E. coli* endonuclease IV (190 ng/µl); 6, Drosophila dUTPase (100 ng/µl). From top to bottom, individual spots represent the application of 2 and 4 µl, respectively, except slot 1 in which 1 and 3 µl were applied.

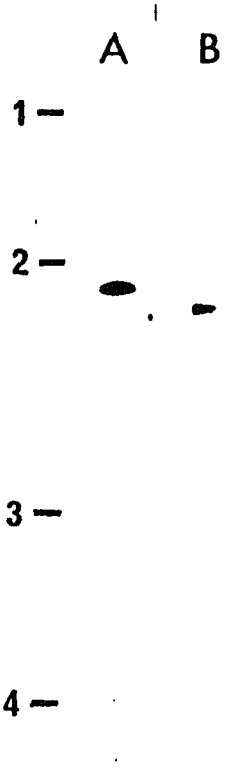


Figure 11. Western blot analysis of AP endonucleases I and II.

Roughly 80 µg of AP endonuclease I, Fraction II, and 60 µg of AP endonuclease II, Fraction II, were subjected in duplicate to SDS-PAGE. One gel was fixed and stained. Proteins were transferred form the other gel onto a nitrocellulose membrane and probed with a 1:50 dilution of rabbit antiserum prepared against HeLa AP endonuclease as described in Materials and Methods. Lane A contained AP endonuclease I and lane B contained AP endonuclease II. Molecular weight markers used were: 1, phosphorylase b (94,000); 2, bovine serum albumin (67,000); 3, ovalbumin (43,000); 4, carbonic anhydrase (30,000).

DISCUSSION

The data presented here show the isolation of two distinct AP endonucleases from Drosophila melanogaster. The two enzymes are easily separated by phosphocellulose chromatography -- AP endonuclease I appears in the column flowthrough and AP endonuclease II elutes at 200-400 mM potassium phosphate. The two enzymes differ in reaction requirements: AP endonuclease II requires divalent cations for activity, with 10 mM $MgCl_2$ giving optimum activity; AP endonuclease I has no apparent requirement for monovalent or divalent cations. AP endonuclease I has a pH optimum of 6.5; AP endonuclease II has an optimum pH of 7.0. Additionally, AP endonuclease II is inhibited by EDTA while AP endonuclease I is not.

Inhibition of both D. melanogaster enzymes by harmane suggests the possiblity of associated DNA-glycosylase activity. Since there does not appear to be any uracil DNA-glycosylase in D. melanogaster (Deutsch and Spiering, 1982), there may be as yet unidentified DNA glycosylase activity associated with the two D. melanogaster enzymes. Adenine containing compounds may inhibit the AP endonucleases to allow for the action of D. melanogaster insertase to repair the apurinic sites (Deutsch and Spiering, unpublished results). Inhibition of AP endonucleases with concomitant stimulation of insertase may be one mechanism for the selection of excision repair over lesion reversion for the repair of AP sites.

AP endonucleases I and II are specific for both apurinic and apyrimidinic baseless sites. Neither enzyme exhibits any endonuclease

activity upon MMS, OsO_4, UV, or single-stranded nontreated DNA. Both enzymes are devoid of any contaminating phosphomonoesterase or exonuclease activities, though it is not certain that the two enzymes are purified to homogeneity. AP endonuclease I fractions consistently contain a co-purifying endonuclease of undetermined specificity. The two nuclease activities in AP endonuclease I fractions appear in a reproducible ratio of 60% AP to 40% NT activity when saturating enzyme levels are used in the standard nuclease assay. AP endonuclease I and this "non-specific" endonuclease may reside on the same or separate polypeptides, as SDS-PAGE analysis of Fraction IV of AP endonuclease I reveals multiple silver-staining bands. Regardless, it has been impossible to separate the two activities.

AP endonuclease II, Fraction V, has been purified so as to lack any non-specific endonuclease activities. Upon SDS-PAGE analyis, AP endonuclease II, Fraction IV, shows multiple bands. The final Fraction V, however, contains protein at a concentration below that detectable by either Coomassie Blue or silver staining.

Glycerol gradient analysis of the two enzymes resulted in estimated molecular weights of less than 40,000 for both enzymes. Conversely, both AP endonucleases I and II elute between the V_o and BSA (67,000) on Sephadex G-100. Utilizing a polyclonal antibody against HeLa AP endonuclease, Western blots of SDS-PAGE were done. Analysis of the resulting autoradiograms suggests a subunit molecular weight for AP endonuclease I of 68,000 and for AP endonuclease II of 64,000. These molecular weight estimates are in close agreement with molecular weight estimates from Sephadex G-100 chromatography. The discrepancy between these molecular weight estimates and the glycerol

gradient estimation of molecular weight may be due to a protein conformation which would cause the proteins to migrate as much smaller proteins on centrifugation in glycerol gradients. The majority of AP endonucleases identified in other organisms have molecular weights between 25,000-40,000 daltons. Only one other AP endonuclease comparable in size to the D. melanogaster enzymes has been isolated to date, the B. subtilis AP endonuclease with a molecular weight of 56,000 (Freidberg et al., 1981). Since the antibody developed against HeLa AP endonuclease only cross-reacted with one band in a heterologous preparation of AP endonuclease I (Fraction IV) and one band in AP endonuclease II (Fraction IV), it is assumed to give a representative subunit molecular weight of the two enzymes.

III. MECHANISMS OF ACTION:

DETERMINATION OF CLEAVAGE PRODUCTS

MATERIALS AND METHODS

Materials. [5-^{3}H]dATP (21.2 Ci/mmol) was purchased from NEN. [γ-^{32}P]ATP (4500 Ci/mmol) and [α-^{32}P]dATP (3200 Ci/mmol) were purchased from ICN. [5-^{3}H]dUTP (14.8 Ci/mmol) was purchased from Amersham. Unlabelled deoxyribonucleosides, uracil, and dAMP were purchased from Sigma.

Enzymes. E. coli polymerase I (5.5 units/µl) was obtained from Bethesda Research Laboratories; one unit of activity is the amount of enzyme necessary to convert 10 nmol of total deoxyribonucleotides to an acid-insoluble form in 30 min at 37°C. Bacterial alkaline phosphatase (30-60 units/mg) was purchased from Worthington. Polynucleotide T4 kinase (10 units/µl) was purchased from Bethesda Research Laboratories; one unit catalyzes the transfer of 1 nmol phosphate from ATP to polynucleotide in 30 min at 37°C. Endonuclease IV and uracil DNA-glycosylase were prepared from E. coli strains deficient for AP endonuclease VI as previously described (Deutsch and Spiering, 1982).

DNA substrates. PM2 DNA and PM2 [^{3}H]DNA were prepared as described in Section II. Depurination, resulting in 1 AP site/genome, was by heating at 70°C, pH 5.2, for 6.5 min. The co-polymer [dAMP-5'-^{32}P, uracil-^{3}H]poly(dA-dT), specific activity 2107 ^{32}P cpm/pmol nucleotide and 6 ^{3}H cpm/pmol nucleotide, was prepared by W. A. Deutsch according to the procedure of Schachman et al. (1960). Depyrimidination was by the action of E. coli uracil DNA-glycosylase for 60 min at 37°C.

DNA synthesis at nicked sites. Reaction mixtures were as described in Section II for nuclease reactions, utilizing unlabelled PM2 DNA. Depurinated PM2 DNA (200 fmol DNA molecules) was incised by either AP endonuclease I, AP endonuclease II, or E. coli endonuclease IV to cleave all available alkali-labile sites per PM2 genome. Parallel nuclease reactions on PM2 [^{3}H]DNA were used to determine the precise number of nicks/genome. The incision reaction was terminated by heat inactivation of the nucleases for 3 min at 70°C. Reaction mixtures (225 µl) were then brought to 70 mM potassium phosphate, pH 7.5/7 mM $MgCl_2$/90 µM each dGTP, dCTP, and TTP/2-3 µCi [^{3}H]dATP and 0.75 unit E. coli polymerase I. Incubation was at 37°C with 50 µl aliquots removed at 0, 10, 20, and 30 min. The [^{3}H]dAMP incorporation was quantitated on Whatman GF/C filters by liquid scintillation (Warner et al., 1980).

Complementation nicking assays. Reaction conditions were as described previously for the apurinic endonuclease assay, with 1 AP site/PM2 genome in a 50 µl reaction volume. Parallel nuclease reactions on PM2 [^{3}H]DNA determined the precise number of nicks/genome for each individual enzyme as well as for the complementation reactions. It was essential that sufficient enzyme be added to the primary reaction mixture to cleave all available AP sites and that the secondary endonuclease reactions create no additional nicks/DNA molecule. Following the primary incision reaction at 37°C, endonucleases were heat-inactivated at 70°C for 3 min and the reaction mixture returned to ice. This same reaction mix was then incised with a second endonuclease at a sufficient concentration to cleave 1 AP site/genome. The reaction was again terminated by heat-treatment for

3 min at 70°C. This final reaction mixture was then utilized as a substrate for DNA synthesis reactions as previously described.

Bacterial alkaline phosphatase (BAP) reactions. Depurinated PM2 DNA (200 fmol DNA molecules, 1 AP site/genome) was incised with an endonuclease under conditions described for nuclease reactions in Section II. Endonucleases were heat-inactivated at 70°C for 3 min, and the reaction put on ice. Following the addition of 3 µl 0.1 M NaOH, either 0.39 unit BAP or deionized water was added to each reaction. BAP was pre-treated at 90°C for 5 min to inactivate any contaminating nucleases (Grafstrom et al., 1982). Reactions with BAP were routinely 30 min at 65°C. The BAP reaction was terminated by the addition of 3 µl 0.12 N HCl to return the pH to the neutral range. Completion of the BAP reactions was followed by DNA synthesis reactions described above. Any effect of the BAP treatment on DNA was determined by monitoring DNA synthesis at the nicked sites against a non-BAP treated reaction.

5'-End labelling at incised sites. Reaction mixtures were as previously described for a nuclease assay, except the reaction was increased 6-fold to 300 µl. PM2 [^{3}H]DNA (240 fmol DNA molecules) was depurinated to contain approximately 1.5 AP sites/genome. Endonuclease activity was terminated by 3 min at 70°C. A 50 µl aliquot was then removed for standard endonuclease activity analysis. The remaining reaction was then divided into two 100 µl aliquots, one of which was BAP-treated as described above. Reaction mixtures (260 µl) were subsequently brought to 2 mM potassium phosphate, pH 6.8/10 mM $MgCl_2$/0.7 mM dithiothreitol/50 mM Tris-HCl, pH 8.2, 3 units T4 kinase and 2.7 pmol [γ-^{32}P]ATP. After 30 min at 37°C, reactions were

put on ice and 0.2 ml 0.1 M $NaPP_i$, 0.1 ml 10 mg/ml BSA, and 0.7% trichloroacetic acid were added. Acid precipitation was for 15 min at 0°C. Samples were filtered through GF/C filters and ^{3}H and ^{32}P quantitated by liquid scintillation.

Near neighbor analysis. The substrate [dAMP-5'-^{32}P, uracil-^{3}H]poly(dA-dT) was depyrimidinated with E. coli uracil DNA-glycosylase to remove approximately 80% total uracil as determined by paper chromatography. Excess AP endonuclease II and E. coli endonuclease IV were allowed to react individually for 30 min and in complementation for an additional 30 min with the co-polymer at 37°C under nuclease assay conditions. Cleavage products, together with the authentic markers uracil and dAMP, were analyzed by paper chromatography as described by Mosbaugh and Linn (1980).

RESULTS

Ability of nicks generated by D. melanogaster AP endonucleases to support DNA synthesis. Class II AP endonucleases have been shown to cleave DNA leaving a deoxyribose 5'-P and a 3'-OH (Fig. 1). This resultant 3'-OH is essential for DNA polymerase to bind to the DNA prior to DNA synthesis. Class I AP endonucleases would be expected to produce termini which support DNA polymerase binding, but subsequent DNA synthesis would proceed at a rate less than 50% that observed for a Class II enzyme due to the slow removal of the remaining 3'-deoxyribose (Warner et al., 1980). Neither Class III or IV AP endonucleases would be expected to support DNA synthesis, due to the presence of a 3'-P.

Figure 2 shows the results of DNA synthesis on depurinated PM2 [^{3}H]DNA incised independently by E. coli endonuclease IV, D. melanogaster AP endonuclease I, or D. melanogaster AP endonuclease II. The amount of DNA synthesis observed for E. coli endonuclease IV agrees well with values reported by Warner et al. (1980) for this Class II enzyme. D. melanogaster AP endonuclease II supports DNA synthesis, but at approximately 30% the level observed for E. coli endonuclease IV. Conversely, cleavage of depurinated DNA by AP endonuclease I supports [^{3}H]dAMP incorporation only to the extent of that observed for reactions in the absence of any endonuclease. These results suggest that D. melanogaster AP endonuclease II cleaves depurinated PM2 DNA producing a 3'-deoxyribose-OH and a 5'-P, a Class I cleavage mechanism. The absolute lack of any DNA synthesis on

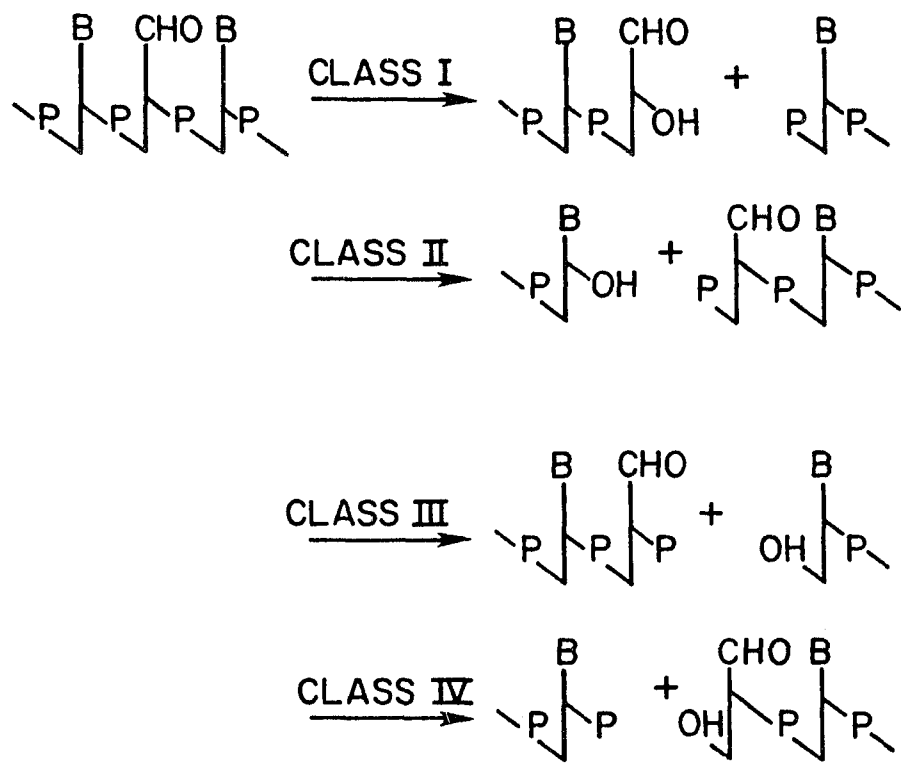


Figure 1. Theoretical cleavage sites for AP endonucleases.

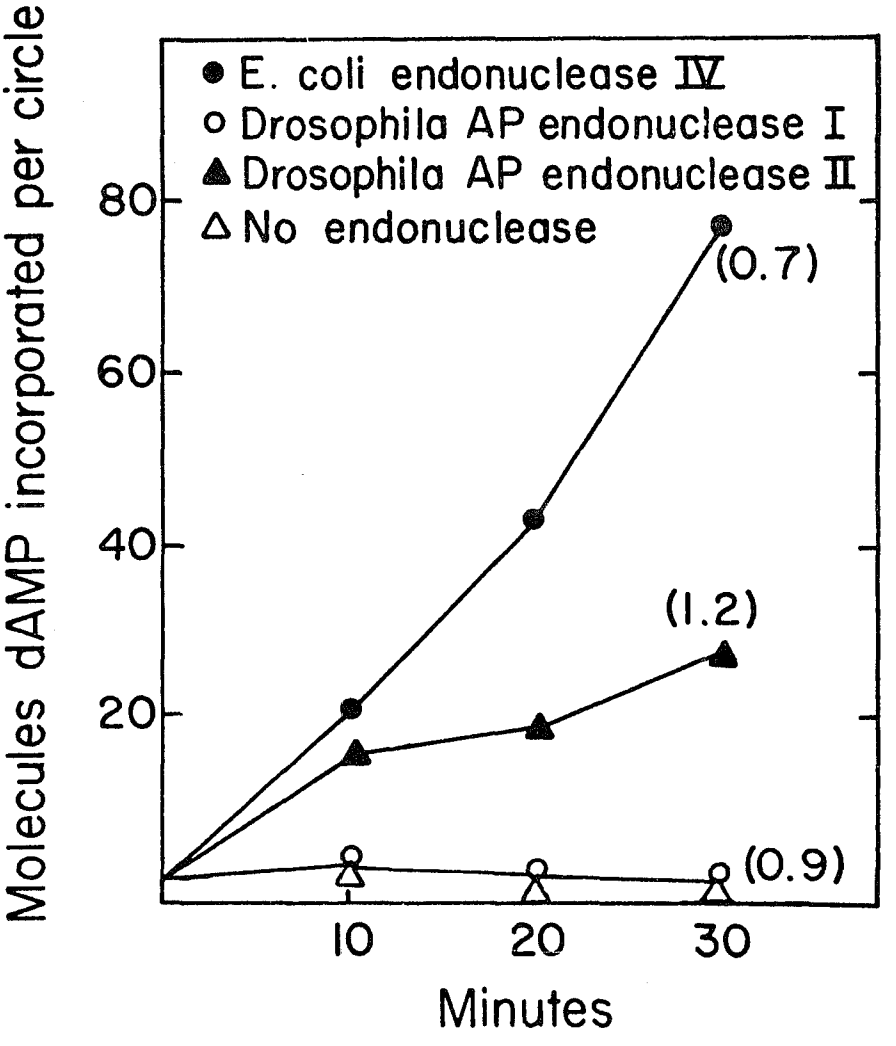


Figure 2. Priming activity of D. melanogaster AP endonuclease I or II and E. coli endonuclease IV.

Partially depurinated DNA (200 fmol DNA molecules) was incubated with no enzyme (Δ), 0.02 unit of AP endonuclease I (O), or 0.03 unit of AP endonuclease II (▲) for 5 min at 37°C or with 0.01 unit E. coli endonuclease IV (●) for 15 min at 37°C. The numbers in parentheses indicate the number of incisions per PM2 [^{3}H]DNA molecule.

termini produced by AP endonuclease I, on the other hand, suggests the possibility of a unique cleavage mechanism. The total lack of any [^{3}H]dAMP incorporation seen with AP endonuclease I cleavage of depurinated DNA would be consistent with the production of a 3'-P terminus (either a Class III or Class IV cleavage).

To determine if the incisions left by the D. melanogaster enzymes could be "activated" to support an increase in DNA synthesis, complementation nuclease reactions were done. (Secondary reactions did not increase the number of nicks per DNA molecule.) As shown in Figure 3, complementation of AP endonuclease I incisions with E. coli endonuclease IV increased DNA synthesis to levels seen with incisions created by E. coli endonuclease IV alone. Likewise, AP endonuclease II cleavage products could be activated by E. coli endonuclease IV to promote increased DNA synthesis. This ability to transform inefficient primers for DNA polymerase I DNA synthesis into efficient primers by a secondary cleavage with a Class II enzyme suggests that both D. melanogaster AP endonucleases cleave on the same side of the AP site, opposite to the side recognized by E. coli endonuclease IV. This was confirmed by the inability of AP endonuclease II/AP endonuclease I complementation to stimulate DNA synthesis above levels seen by AP endonuclease II alone (Fig. 3). These results, combined with the individual DNA synthesis reaction results, imply that D. melanogaster AP endonuclease I is a Class III enzyme and AP endonuclease II is a Class I enzyme.

Activation of DNA synthesis by BAP treatment. The inability of AP endonuclease I cleavage to support any DNA synthesis, whatsoever, suggests the production of a 3'-P upon DNA cleavage. If correct, the

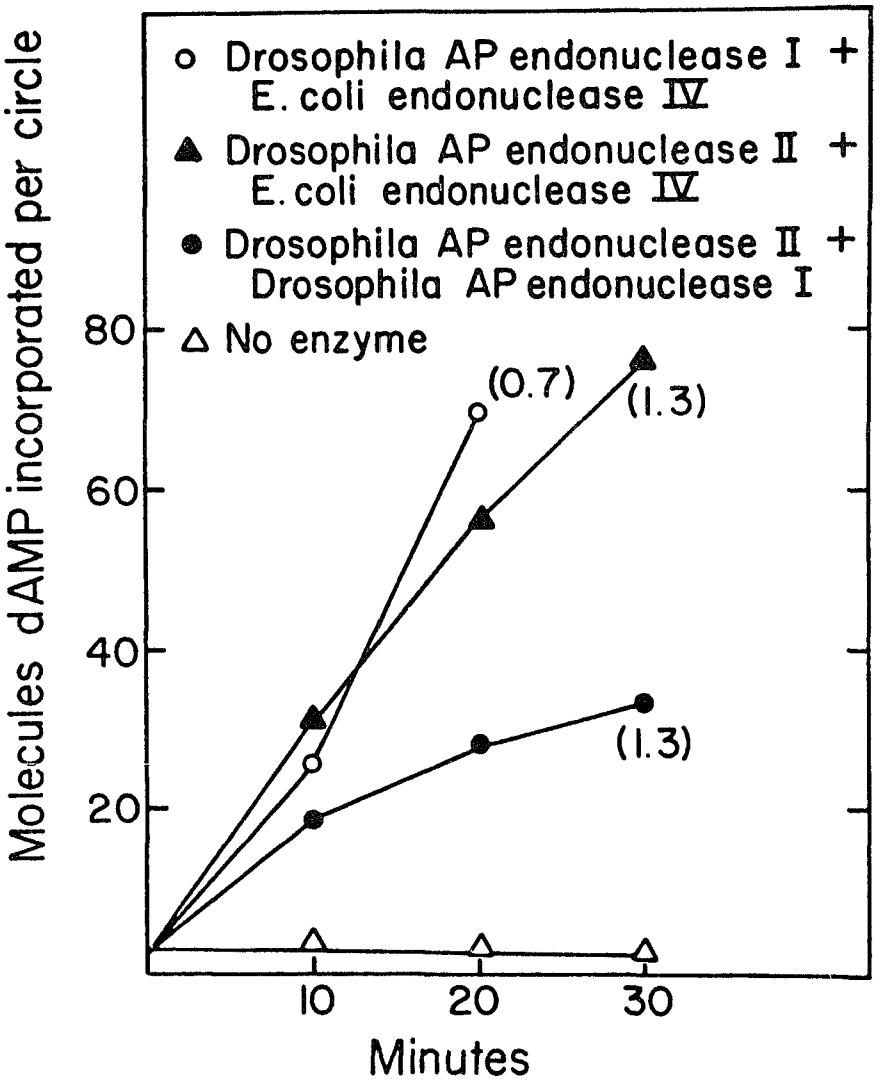


Figure 3. E. coli endonuclease IV activates incision sites produced by D. melanogaster AP endonuclease I or II.

Primary reactions were identical to those described in Figure 2. The reactions were terminated by heating to 70°C for 3 min, then E. coli endonuclease IV (0.01 unit) was added for an additional 15 min at 37°C or AP endonuclease I (0.03 unit) was added for 5 min at 37°C.

treatment of AP endonuclease I incisions with BAP should result in an increase of DNA synthesis above that seen for AP endonuclease I alone. This in fact was observed (Fig. 4). E. coli endonuclease IV products treated with BAP, as expected, did not stimulate DNA synthesis above levels seen with endonuclease IV alone. This activation of DNA cleaved by AP endonuclease I by BAP-treatment reinforces the suggestion that AP endonuclease I cleavage of depurinated DNA results in the production of a 3'-phosphate terminus, rather than a 3'-OH terminus. Again, these results suggest a Class III mechanism for D. melanogaster AP endonuclease I.

5'-End labelling at incised sites. The preceding experiments imply that AP endonuclease I is a Class III enzyme, leaving deoxyribose-3'-P and nucleotide-5'-OH termini upon cleavage. T4 polynucleotide kinase is capable of phosphorylating a 5'-nucleotide-OH terminus, but not a 5'-deoxyribose-OH terminus (Hadi et al., 1973). If the nucleotide terminus generated by AP endonuclease I contains a 5'-OH, it should be phosphorylated without prior phosphodiesterase treatment. E. coli endonuclease IV is used as a negative control -- as a Class II enzyme it should not be susceptible to phosphorylation by T4 kinase. E. coli endonuclease IV cleavage products are not phosphorylated by T4 polynucleotide kinase above the level seen for non-incised DNA (Table I, experiment 1). Conversely, the cleavage products of AP endonuclease I (phosphocellulose fraction) were susceptible to phosphorylation by T4 polynucleotide kinase, either with or without phosphodiesterase treatment. This strongly suggests that AP endonuclease I leaves a 5'-nucleotide-OH terminus, i.e., a Class III cleavage event. D. melanogaster AP endonuclease II

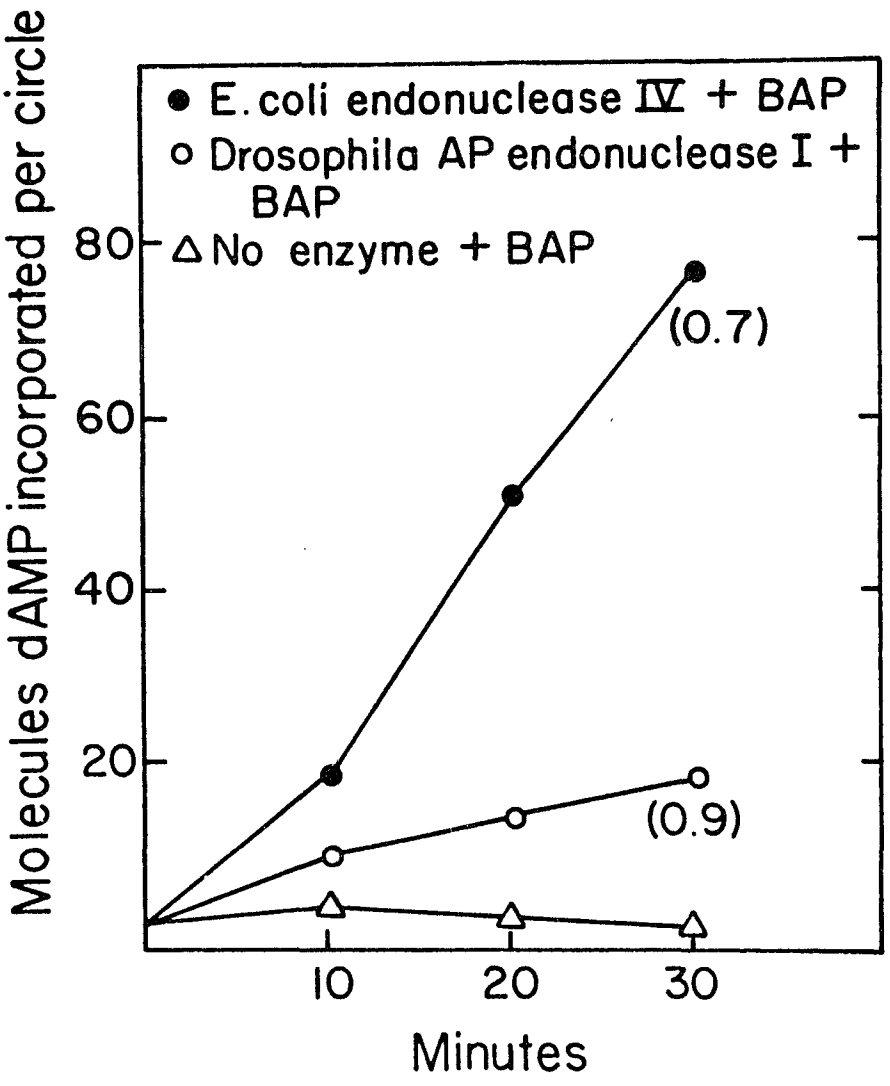


Figure 4. Activation by phosphomonoesterase of incision sites produced by AP endonuclease I.

Primary reactions were described in Figure 2. Conditions for BAP treatment were described in Materials and Methods.

Table I. 5'-End labelling of depurinated DNA cleaved by D. melanogaster AP endonucleases of E. coli endonuclease IV.

Reactions for Experiment 1 contained 0.07 unit AP endonuclease I, 0.03 unit AP endonuclease II, or 0.004 unit E. coli AP endonuclease IV, and 200 fmol [^{3}H]DNA molecules. For Experiment 2, 0.7 unit AP endonuclease I, 5.6 units AP endonuclease II, or 0.6 unit E. coli endonuclease IV, and 83 fmol [^{3}H]DNA molecules were used.

Experiment	Addition	nicks introduced per DNA molecule	Phosphatase Treatment	
			-	+
			fmol ^{32}P incorporated	
1	No enzyme	0.2	1.0	1.4
	AP endonuclease I	1.4	29.2	26.4
	AP endonuclease II	1.3	6.8	5.2
	E. coli endonuclease IV	1.4	0.3	0.6
2	No enzyme	0.2	0.2	
	AP endonuclease I	1.2	21.0	
	AP endonuclease II	1.7	0.3	
	E. coli endonuclease IV	1.5	0.3	

(phosphocellulose fraction) shows slight phosphorylation (5% total incisions phosphorylated) either with or without BAP treatment. To ascertain if this phosphorylation was due to the actual incision termini or to some associated activity (exonuclease or phosphomonoesterase), kinase treatment was repeated using more purified fractions of each D. melanogaster enzyme (Table I, experiment 2). The levels of phosphorylation of E. coli endonuclease IV and D. melanogaster AP endonuclease I cleavage termini remain at levels comparable to experiment 1. However, utilization of a Sephadex G-100 fraction of AP endonuclease II resulted in a level of phosphorylation comparable to E. coli endonuclease IV alone or the non-incised DNA control. From these series of experiments, one may conclude AP endonuclease I is a Class III enzyme. D. melanogaster AP endonuclease II incisions are not phosphorylated either with or without BAP-treatment. Phosphorylation in the absence of BAP-treatment would not be expected for a Class I AP endonuclease. However, one would expect to see slight phosphorylation after phosphatase treatment for a Class I incision. The lack of ^{32}P incorporation seen with AP endonuclease II is presumably due to the incomplete inactivation of the phosphatase, as the level of ^{32}P incorporated in all synthesis reactions with BAP pre-treatment is slightly less than that seen in control reactions. If the BAP were not totally inactivated, this decrease could result from the BAP reacting with the deoxyribonucleotides present (only 9 μM) in the DNA synthesis reaction.

Near neighbor analysis of cleavage products. To directly determine the termini resulting from AP endonuclease II cleavage, the synthetic co-polymer [dAMP-5'-^{32}P,uracil-^{3}H]poly(dA-dT) was used as a

substrate. This substrate contains regions of [^{3}H]dUMP in place of dTMP and ^{32}P-label at the 5'-phosphate to dAMP (Fig. 5). The generation of AP sites was by depyrimidination using E. coli uracil DNA-glycosylase. This treatment resulted in the removal of 80% [^{3}H]uracil as determined by paper chromatography. Exposure of the depyrimidinated co-polymer to excess D. melanogaster AP endonuclease II or excess E. coli endonuclease IV did not result in a substantial release of [^{32}P]dAMP (Table II). However, complementation of AP endonuclease II cleavage with E. coli endonuclease IV resulted in the appearance of 125 pmol [^{32}P]dAMP. The production of [^{32}P]dAMP from the joint cleavage suggests AP endonuclease II cleaves 3' to the AP site, leaving a 3'-OH. Treatment of this product with a secondary incubation using a Class II endonuclease is the only possible way free dAMP may be generated from this substrate. The orignal substrate contained 334 pmol AP sites, with 125 pmol of [^{32}P]dAMP being produced by the combined action of both AP endonucleases. Not all the ^{32}P would be expected to be recovered as [^{32}P]dAMP, since the production of labelled dAMP should only occur when flanked by AP sites.

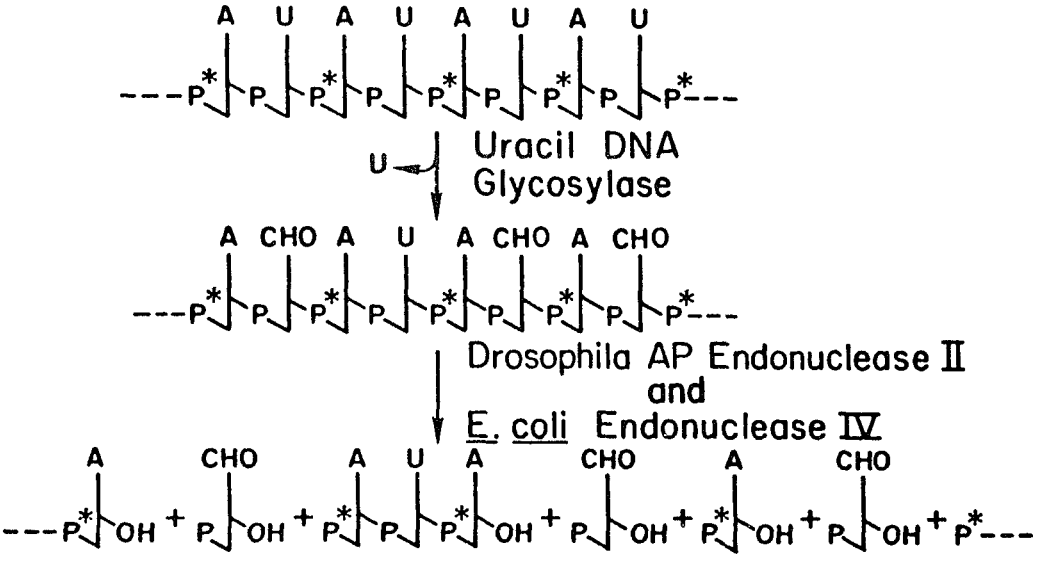


Figure 5. Model for the digestion of poly (dA-dU) sequences. The position of the ^{32}P-label is indicated by $^{*}P$.

Table II. Isolation of dAMP from partially depyrimidinated DNA after incision by D. melanogaster AP endonuclease II and E. coli endonuclease IV.

Reaction mixtures (50 µl) contained 25 mM Tris-HCl (pH 7.0), 0.4 nmol [dAMP-5'-^{32}P,uracil-^{3}H]poly(dA-dT), and uracil-DNA glycosylase. After depyrimidination, incubations continued with the addition of $MgCl_2$ (10 mM) and 9.9 units AP endonuclease II for 30 min or 11 units E. coli endonuclease IV for 30 min at 37°C. A second incubation for 30 min at 37°C with 11 units E. coli endonuclease IV followed heat inactivation of primary reactions with AP endonuclease II. The products were evaluated by descending chromatography.

Addition	Product recovered	
	[^{3}H]uracil	[^{32}P]dAMP
	pmol	pmol
No enzyme	318	
AP endonuclease II	389	38
E. coli endonuclease IV	318	27
AP endonuclease II/ E. coli endonuclease IV	334	125

DISCUSSION

The results presented here demonstrate the presence of two different mechanistic classes of AP endonuclease in D. melanogaster. AP endonuclease I, the phosphocellulose flowthrough activity described in Section II, is a Class III enzyme. AP endonuclease II, retained by phosphocellulose as described in Section II, is defined as a Class I enzyme. These conclusions are substantiated by several observations. Neither D. melanogaster AP endonuclease is capable of supporting DNA synthesis at the level of E. coli endonuclease IV, a known Class II enzyme. The incision of depurinated DNA by AP endonuclease I appears totally incapable of supporting DNA synthesis by DNA polymerase I, whereas AP endonuclease II supports less than 50% the amount of [^{3}H]dAMP incorporation demonstrated by a Class II enzyme. Additionally, both enzymes may be complemented with E. coli endonuclease IV to promote increased DNA synthesis without an increase in nicks per genome. Conversely, no increase in DNA synthesis is seen by complementation of AP endonuclease II with AP endonuclease I. This suggests both D. melanogaster AP endonucleases cleave 3' to the AP site. AP endonuclease II appears to leave a 3'-deoxyribose-OH to allow DNA polymerase I binding, but inefficient DNA synthesis due to the slow removal of the 3'-deoxyribose. AP endonuclease I leaves a 3'-P, accounting for the total lack of observable DNA synthesis.

To confirm the cleavage products of AP endonuclease I, incision products were pre-treated with bacterial alkaline phosphatase prior to DNA synthesis. This was found to activate incisions, thus supporting

the model of a Class III mechanism for AP endonuclease I. 5'-End labelling additionally supports the contention that AP endonuclease I is a Class III enzyme.

The determination of D. melanogaster AP endonuclease I as a Class III enzyme is a unique observation. No other organism, prokaryotic or eukaryotic, has been reported to contain nucleases capable of cleaving depurinated DNA to produce a 3'-deoxyribose-P and 5'-OH termini. Secondly, it is the first report of a eukaryote expressing a Class I AP endonuclease without a concurrent Class II enzyme. The inefficient primer termini produced by Class I enzymes have been believed to be complemented by the action of Class II enzymes to leave a one-nucleotide gap in the DNA. The lack of concomitant Class II AP endonucleases in D. melanogaster may exemplify a unique pathway for the repair of AP sites in DNA. It is possible that D. melanogaster require the action of phosphomonoesterases and/or exonucleases following AP endonuclease cleavage prior to DNA repair synthesis.

Alternately, AP endonuclease I, the Class III enzyme, might not be involved in DNA repair at all. Rather, the associated nonspecific nuclease and the AP endonuclease could be responsible for the degradation of DNA during the normal developmental sequence of the organism. D. melanogaster AP endonuclease II, the Class I enzyme, may be the only DNA repair AP endonuclease as it produces a more efficient primer terminus for DNA synthesis. Analysis of base excision repair deficient D. melanogaster strains should provide an insight into the possible roles of a Class I and a Class III AP endonuclease in Drosophila melanogaster.

IV. THE GENETICS OF AP SITE REPAIR

MATERIALS AND METHODS

Drosophila melanogaster strains. The wild-type strain Oregon-R was utilized as the control for basal AP endonuclease activity. Two mutagen sensitive strains were used, mei-9^a and mus(2)201. Table I lists the pertinent characteristics of each strain. Mutagen sensitive stocks were routinely tested for MMS-sensitivity by Dr. P. Foureman to ensure stocks to be repair deficient. All D. melanogaster stocks were maintained in the laboratory of Dr. W. R. Lee, Dept. of Zoology, LSU.

Dot blot analysis of nitrocellulose-bound proteins. The procedure of Burnette (1981) was followed as described in Section II.

Purification of AP endonucleases from adult D. melanogaster. AP endonucleases I and II were purified through phosphocellulose chromatography essentially as described in Section II. Adult organisms were used in place of embryos as the mutant stocks exhibit decreased fecundity, hampering collection of large quantities of mei-9^a or mus(2)201 embryos. AP endonuclease activity was analyzed using the standard nuclease assay described in Section II. If no AP endonuclease was detectable by the standard nuclease assay, fractions corresponding to the conductivity at which the wild-type AP endonuclease enzymes would be expected to elute were pooled for immunological analysis.

Other methods. D. melanogaster extracts were concentrated by centrifugation in centricon-10 concentrators at 5500 rpm in a Beckman JA-21 rotor. Protein determination followed the method of Bradford (1976), utilizing BSA as the standard.

Table I. Characteristics of three D. melanogaster strains.

Characteristic	D. melanogaster Strain		
	Oregon-R	mei-9[a]	mus(2)201
locus		1-6	2-23
sensitivity to:[1]			
X-ray	normal	v.sens.	sens.
UV	normal	v.sens.	v.sens.
MMS	normal	v.sens.	v.sens.
Post-replication repair[2]	normal	normal	normal
Base excision repair[2]	normal	deficient	deficient
% Hatching[1]	93	20	72

Legend: Normal denotes the wild-type condition; v.sens.: very sensitive compared to the wild-type; sens.: sensitive compared to the wild-type.

[1] Smith et al., 1980

[2] Boyd et al., 1980

RESULTS

Detection of AP endonucleases I and II by standard nuclease assay. Both AP endonuclease I and II from adult wild-type D. melanogaster eluted from phosphocellulose in positions corresponding to those described in Section II for the embryonic enzymes. Adult mus(2)201 D. melanogaster also express both AP endonucleases I and II; mei-9^a adult stocks, however, lack detectable AP endonuclease II activity (S. Venugopal and W. A. Deutsch, unpublished results).

Dot blot analysis of adult Drosophila stocks. To determine if mei-9^a adult D. melanogaster produce an AP endonuclease II protein, either in amounts undetectable via the nuclease assay or in an inactive form, dot blot analysis was done. Figure 1 depicts the cross-reaction of an antibody, prepared toward a human AP endonuclease, against D. melanogaster wild-type, mus(2)201, and mei-9^a phosphocellulose extracts. Wild-type adult flies produce AP endonucleases I and II in amounts sufficient to cross-react with the antibody toward HeLa AP endonuclease. AP endonuclease I from mus(2)201 cross-reacts; mus(2)201 AP endonuclease II, however, does not appear to cross-react with the polyclonal antibody. Similarly, the mei-9^a extracts exhibit cross-reactivity between AP endonuclease I and the anti-HeLa AP endonuclease, but no cross-reactivity is seen with AP endonuclease II. The lack of cross-reactivity between mei-9^a extracts for AP endonuclease II and the HeLa AP endonuclease antibody were presupposed by the absence of any detectable AP endonuclease II activity in phosphocellulose fractions. The mus(2)201 stocks,

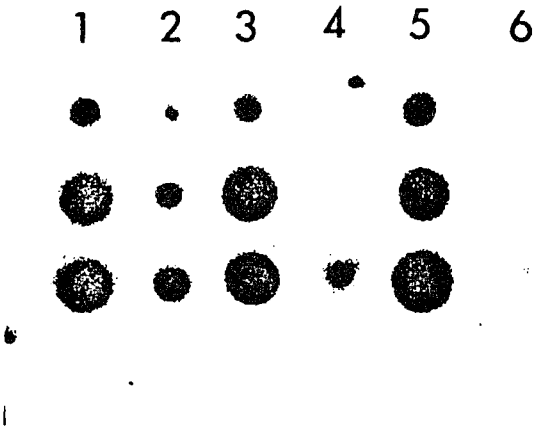


Figure 1. Initial analysis of mutagen sensitive stocks: antibody reaction with nitrocellulose-bound protein.

Proteins applied to a nitrocellulose filter were: 1, wild-type AP endonuclease I, 172 ng/µl; 2, wild-type AP endonuclease II, 125 ng/µl; 3, mei-9^a AP endonuclease I, 104 ng/µl; 4, mei-9^a AP endonuclease II, <40 ng/µl; 5, mus(2)201 AP endonuclease I, 88 ng/µl; 6, mus(2)201 AP endonuclease II, 25 ng/µl. From top to bottom, individual spots represent the application of 1, 3, and 5 µl.

however, do contain AP endonuclease II activity. To determine if the lack of antibody-antigen complex formation was due to a low protein concentration or to an alteration in antigenic determinants, the extracts were concentrated and the analysis repeated.

Using higher protein concentrations, a second dot blot (Fig. 2) produced similar results to those seen in Figure 1. Neither the mus(2)201 or mei-9^a phosphocellulose high-salt extracts exhibited any cross-reactivity with the polyclonal antibody against HeLa AP endonuclease. These results imply that the decrease in base excision repair in the two mutant stocks may be due to an alteration in the AP endonuclease II protein.

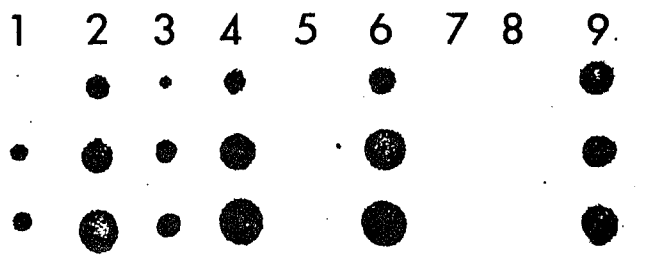


Figure 2. Analysis of mutagen sensitive D. melanogaster strains by antibody reaction.

Proteins applied to a nitrocellulose filter were: 1, wild-type AP endonuclease I, 70 ng/µl; 2, wild-type AP endonuclease I, 172 ng/µl; 3, wild-type AP endonuclease II, 125 ng/µl; 4, mei-9^a AP endonuclease I, 104 ng/µl; 5, mei-9^a AP endonuclease II, 147 ng/µl; 6, mus(2)201 AP endonuclease I, 88 ng/µl; 7, mus(2)201 AP endonuclease II, 82 ng/µl; 8, blank; 9, E. coli endonuclease IV, 190 ng/µl. Individual spots represent the application of 1, 3, and 5 µl from top to bottom.

DISCUSSION

Biochemically, the mus(2)201 and mei-9^a D. melanogaster strains are deficient for base excision repair; the wild-type strain is not. Adult wild-type D. melanogaster exhibit both AP endonucleases I and II. The mus(2)201 stocks express detectable levels of both enzymes, whereas the mei-9^a stocks appear to be deficient in AP endonuclease II activity. To determine if the absence of detectable AP endonuclease II was due to an alteration in the protein structure or an absence in enzymatic activity, immunological analysis was undertaken. Phosphocellulose high-salt extracts from mei-9^a adult D. melanogaster showed no cross-reactivity with the antibody toward HeLa AP endonuclease. This correlates well with the lack of enzymatic activity. Surprisingly, however, phosphocellulose high-salt fractions from mus(2)201, containing AP endonuclease II activity, also showed no cross-reactivity with the antibody. It may well be that the AP endonuclease II protein synthesized by mus(2)201 D. melanogaster strains has been altered such that the antigenic determinant is no longer the same. Since this antigenic determinant has been well conserved through evolution, appearing in E. coli, D. melanogaster, and humans (see Section II, Fig. 10), this alteration may be tied to the increased mutagen sensitivity of this stock.

However, the loss of antigenic determinants on a particular protein is usually due to a large change in the protein structure. It is possible that the adult form of AP endonuclease II is not identical to the embryonic form characterized in Section II. Further

biochemical analysis of the two adult D. melanogaster AP endonucleases may explain the apparent lack of wild-type AP endonuclease II in these two base excision repair deficient D. melanogaster strains. It is plausible that any change in the Class I AP endonuclease could account for the increased sensitivity of both mei-9^a and mus(2)201 strains to MMS, UV, and X-ray. A precedent for this is seen in human cell lines derived from xeroderma pigmentosum Class D patients (Kuhnlein et al., 1978). Base excision competent human cell lines produce both a Class I and a Class II AP endonuclease. These base excision defective cell lines contain only the Class II AP endonuclease activity, completely lacking any detectable AP endonuclease Class I activity. This implies that the presence of a Class I AP endonuclease is extremely important for the retention of genomic integrity via base excision repair. The presence of a Class III AP endonuclease in D. melanogaster (AP endonuclease I) may have no bearing on base excision repair at all. Only if mutagen sensitive strains are identified which are deficient in this activity, yet possess Class I AP endonuclease activity, can one demonstrate Class III AP endonuclease involvement in DNA repair.

The results presented here reveal the importance of a combined biochemical and genetic approach to the complex field of DNA repair. Based on an understanding of AP endonucleases in wild-type Drosophila melanogaster, one can move on to analyze D. melanogaster stocks which may exhibit deficiency or alterations in one or both of the enzymes. This initial investigation of AP endonucleases in the mei-9^a and mus(2)201 strains reveals the sensitivity of such an approach.

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1. Deutsch, W.A., A.L. Spiering and G.R. Newkome. 1980. An *in vitro* characterization of interstrand cross-links in DNA exposed to the antitumor drug *cis*-dichlorodiammineplatinum(II). Biochem. Biophys. Res. Comm. *97*, 1220.

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DOCTORAL EXAMINATION AND DISSERTATION REPORT

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Major Field: Biochemistry/Genetics

Title of Dissertation: The Enzymology and Genetics of DNA Repair in *Drosophila melanogaster*

Approved:

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